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## I. Claim Objection

Applicant acknowledges that claim 80 has been renumbered as claim 68 and such amendment to claim 80 has been made on the attached "Claim Sheets marked-up to show changes" and "Clean Set of Claims."

## II. The Section 112, Second Paragraph Rejection

Claims 1-19 and 68 are rejected by the Examiner under 35 U.S.C. 112, second paragraph as "unclear in the recitation of a 'biological agent'." The claims have been amended to substitute the term "antibody or antigen binding portion thereof" for the term "biological agent," without prejudice to pursue the "biological agent" claims in a subsequent case. Applicant respectfully asserts that this amendment obviates the Examiner's rejection on this ground.

Claims 7-9 are rejected by the Examiner as "unclear in the recitation 'wherein an antibody is used in carrying out said method." Applicant has amended claim 7, which now depends on claim 1 and asserts that it is clear to what the recitation of "antibody" refers.

Claims 12-14 are rejected by the Examiner as "unclear in the recitation of a 'substance effective to kill or ablate." Applicant respectfully disagrees and refers the Examiner to the specification including at page 26, line 1 to page 28, line 31. Here suitable substances that would be "effective to kill or ablate" "normal, benign hyperplastic, and cancerous prostate epithelial cells" are described, but the invention in contemplated to include any such substances. Claims 12-14 are therefore clear and this ground for rejection is respectfully traversed.

## III. The Section 112, First Paragraph Rejection

Claim 80 (now claim 68) is rejected by the Examiner as "containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed

invention." Applicant respectfully disagrees and directs the Examiner to page 29, lines 11-15 wherein "a method of killing or ablating which involves using the biological agents for prophylaxis" is disclosed. Accordingly, Applicant asserts that the invention of claim 68 is "supported by the original disclosure as filed" and does not represent new matter.

Claims 1-19 and 68 are rejected under 35 U.S.C. 112, first paragraph "as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains or with which it is most nearly connected, to make and/or use the invention." The Examiner asserts that "the specification fails to provide sufficient guidance and objective evidence to enable one skilled in the art to predictably kill or ablate cells, including in vivo treatment, using agents or antibodies that bind the extracellular domain of PSMA and target tumor vasculature"; the "instant demonstration that new antibodies E99, J415, J533, and J591 bind to vasculature in fixed tissue does not render it predictable that the new antibodies are binding to PSMA..."; "it is unpredictable and would require undue experimentation to bind agents to PSMA which are also internalized"; and the specification "provides insufficient objective evidence that antibodies to the PSMA extracellular domain, or antibodies E99, J415, J533, and J591 effectively bind to normal, benign hyperplastic and cancerous prostate epithelial cells *in vivo*" and cites Jain, R. R., et al. As detailed below, Applicant respectfully traverses these objections.

**A. The Method to Prepare Antibodies Described in the Application that bind to the External Domain of PSMA is Reproducible.**

As asserted successfully during prosecution of the allowed parent application (U.S. Patent No. 6,107,090) of the instant application, the specification of the application describes a novel method whereby one skilled in the art could reproducibly prepare the monoclonal antibodies of the present invention which bind to the extracellular domain of PSMA present as an integral membrane

protein of a living cell. As developed below, Applicant's use of the same method to generate four (4) different antibodies from two completely separate immunization and fusion experiments is compelling evidence of enablement.

The procedures for producing these antibodies are fully described in the examples of the present application. In particular, the four antibodies described in the application (i.e. E99, J415, J533 and J591) were prepared as set forth in Example 3. Specifically, BALB/c mice were immunized intraperitoneally with viable unmodified LNCaP cells at the specified intervals, followed by a final booster immunization with fresh prostate cells. Spleen cells from these mice were then fused with myeloma cells. Next, appropriate clones producing antibodies to the extracellular domain of PSMA were selected from those producing antibodies to other LNCaP-related antigens. This was accomplished by a screening process again using viable cells known to be PSMA-positive (i.e. LNCaP and prostate cells) or PSMA-negative (i.e. cultured normal kidney and colon cells). Those clones which were LNCaP+/cultured normal kidney -/ colon – and prostate + were further screened by the ability to immunoprecipitate and/or western blot a molecular species comparable to the 7E11 antibody as described in Examples 7 and 8. Reactivity with the external domain of PSMA was further confirmed as described in Example 9 (reactivity with viable LNCaP cells) and the results of the reactivity with normal and cancer tissues, is presented in Table 3.

In fact, Applicant used this same approach more than once to generate the four different antibodies described in the application. (**Bander Declaration #1, ¶7.**) Specifically, antibody E99 is derived from a completely different immunization/fusion experiment than the remaining antibodies denoted by the "J" prefix, i.e. J415, J533 and J591. (Id.) The fact that Applicant was able to use this procedure to obtain 4 different antibodies, with 2 completely separate immunization and fusion experiments, demonstrates that the disclosed procedure can be repeated and used to develop

additional antibodies. Furthermore, procedures for sequencing the variable regions of J591 antibody, while not essential, are set forth in Example 12. Using these procedures, as well as the sequences themselves (which are suitable for hybridization assays), one of ordinary skill in the art would be fully able to identify additional binding portions. Accordingly, Applicant submits that the present application does provide enabling support to reproducibly obtain the antibodies of the claimed invention.

**B. The Antibodies of the Present Invention Bind to the Extracellular Domain of PSMA.**

**1. The Specification Provides Evidence that the Monoclonal Antibodies of the Present Invention Bind to the Same Antigen (although a different epitope) as the 7E11 Antibody (i.e. PSMA) and Bind to the Extracellular Domain of PSMA.**

The Examiner's rejection on the grounds that the present application does not demonstrate that antibodies in accordance with the present invention bind to PSMA (the same antigen bound by 7E11) or an external region of PSMA is respectfully traversed. As noted at page 9, lines 3-9 of the specification, biochemical characterization and mapping have shown that the epitope or antigenic site to which the 7E11 antibody binds is present on the intracellular portion of the PSMA molecule. (**Exhibit 3:** Troyer et al., "Biochemical Characterization and Mapping of the 7E11-C5.3 Epitope of the Prostate-specific Membrane Antigen," Urol. Oncol., 1:29-37 (1995) ) 7E11, as a result of its specificity for the intracellular domain, does not bind to viable LNCaP cells. (**Exhibit 4:** Troyer et al., "Location of prostate-specific membrane antigen in the LNCaP prostate carcinoma cell line," Prostate, 30:232-242. (1997)) Binding of the antibodies of the present invention to the same antigen as 7E11 and the external domain of PSMA is demonstrated by the examples of the present application. In Example 7, the immunoprecipitation and sequential immunoprecipitation studies confirm reactivity of E99, J415, J533 and J591 to PSMA. Specifically, the sequential



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immunoprecipitation study showed that these antibodies and 7E11 bind to the same molecule, i.e. PSMA. In addition, Example 8 of the present application shows that the 7E11 antibody and the antibodies of the present invention precipitate the same band in a Western Blot analysis. Since the 7E11 antibody is specific to PSMA, the claimed antibodies must also bind to PSMA. Moreover, the immunoflorescence against viable LNCaP cells described in Example 7 demonstrates that the antibodies bind to the external domain of PSMA. The results of Examples 9 and 11 as well as Figures 1-4 provide further support for binding of the antibodies of the present invention to the external domain of PSMA.

**2. Further Evidence that the Monoclonal Antibodies of the Present Invention Bind PSMA, the Same Antigen Bound by 7E11.**

The ability of the antibodies of the present invention bind to PSMA as described in the application is confirmed by the peer reviewed article Liu, et. al., "Monoclonal Antibodies to the Extracellular Domain of Prostate-specific Membrane Antigen also React with Tumor Vascular Endothelium," Cancer Res. 57:3629-34 (1997) ("Lui I") (attached hereto as **Exhibit 5**), which was senior authored by the inventor, Dr. Neil Bander. In Figure 1 on page 3630 of Liu I, a cross-immunoprecipitation experiment demonstrates that when one uses the 7E11 antibody to immunoprecipitate its antigen (i.e. PSMA) from LNCaP cells, and when this immunoprecipitated material is transferred to a Western Blot and probed with the antibodies of the present invention, those 4 antibodies react with the material immunoprecipitated by 7E11. The same is true in reverse – i.e., when a LNCaP lysate is immunoprecipitated individually by each of the 4 antibodies of the present invention, a Western Blot with 7E11 reveals that 7E11 is immuno-reactive with the immunoprecipitated species.

Figure 5 of Lui I provides further evidence of the ability of the antibodies of the present invention to bind the same PSMA antigen as 7E11. This figure demonstrates a competition binding assay comparing all 4 antibodies of the present invention to define which of these antibodies bind to the same or different epitopes of PSMA. Key to the issue here is that the technique, which is described on page 3630, first captures PSMA using the 7E11 antibody. The actual competition part of the assay is done after the capture step. This indicates that the 4 antibodies of the present invention bind to PSMA captured by 7E11, again indicating all of these antibodies bind to the same molecule.

Moreover, it has been shown by investigators at Memorial Sloan-Kettering Cancer Center including Chang et al. that the PC3 cell line (which under normal circumstances is PSMA-negative) becomes immuno-reactive with 7E11 as well as the antibodies of the present invention after it has been transfected with the PSMA gene. (**Exhibit 6:** Chang et al., Cancer Res., 59:3192-3198 (1999)). And, Applicant performed a sequential immunoprecipitation analysis that confirms binding of PSMA by the antibodies of the present invention. ("Lui I" at page 3631.) This study demonstrates that a LNCaP lysate, when first "precleared" with any one of the antibodies of the present invention, is no longer immuno-reactive with 7E11. And again the converse is true.

### 3. Further Evidence that Monoclonal Antibodies of the Present Invention Bind to Extracellular Domain of PSMA

Lui I also confirms that the antibodies of the present invention bind to the external domain of PSMA. (See Lui I's immunoflourescence microscopy targeting of living prostate cancer cells (i.e. LNCaP cells) on pages 3632, left column, and Figure 3.) The ability of the antibodies of the present invention to bind to viable, non-permeabilized LNCaP cells (Figure 3, panels A,C,E and G) demonstrates that the binding occurs on the extracellular region of the PSMA molecule. On the

other hand, the 7E11 antibody, which is known to bind to an intracellular epitope, does not bind to such viable cells (Figure 3, panel I). Only when LNCaP cells were permeabilized could the 7E11 antibodies (as well as the 4 antibodies of the present invention) react with these cells. Furthermore, as depicted in the immunoelectron micrograph of Figure 4 of Liu I, viable LNCaP cells were incubated with the J591 antibody of the present invention. As Figure 4 visually demonstrates, J591 localizes to the extracellular surface of the plasma membrane, (Panel A) whereas 7E11 demonstrates no binding (Panel B).

Further evidence that the antibodies of the present invention bind to the extracellular domain of PSMA is provided in Liu, et al., "Constitutive and Antibody-Induced Internalization of Prostate-Specific Membrane Antigen," Cancer Res. 58:4055-60 (1998) ("Lui II") (attached hereto as **Exhibit 7**) and also senior-authored by the inventor, Dr. Neil Bander. In particular, in Figure 1 of Lui II on page 4056, a time course study of the antibodies of the present invention show antibodies binding to viable LNCaP cells. In this experiment, the antibodies can first be seen binding to the plasma membrane and subsequently being internalized. Figure 3 of Lui II shows similar data at the level of the electron microscope.

Based upon all this evidence, it is clear that the antibodies of the present invention bind to an extracellular antigen of PSMA.

**C. In vivo Data Demonstrates that the Antibodies of the Present Invention Successfully Ablate or Kill Tumor Tissue.**

**1. The Jain Factors are not Relevant**

The Examiner questions the evidence presented that the antibodies of the present invention effectively target tumors *in vivo*. The Examiner cites Jain and three factors responsible for poor localization of macromolecules in tumors: (1) heterogeneous blood supply; (2) elevated interstitial

pressure; and (3) large transport distances. Applicant respectfully submits that these three factors are not relevant to the clinical situation addressed by the present invention. First, prostate cancer predominantly involves the bone marrow and lymph nodes. These sites are, by definition, highly vascularized. (**Bander Declaration #2, ¶ 9.**) In addition, prostate cancer is unlike many other solid tumors in that its metastatic sites are small volume sites measure in microns or millimeters. (Id.) For these reasons, the tumor sites are very well supplied with antibody given the high levels of circulating antibody in the vascular compartment. Large transport distances, as a result, are not operative in this situation. (Id.) Similarly, interstitial pressure in these small volume sites within bone marrow and lymph nodes is also not operative. (Id.) Moreover, primary prostate cancers are also relatively small and multifocal and the factors recited by the Examiner are likewise not relevant. (Id.)

With respect to targeting the PSMA molecule on vascular endothelial cells, these three factors cited by the Examiner similarly are not operative. (**Bander Declaration #2, ¶10.**) Since blood supply is necessary to allow tumor nutrition and growth, and since the method of the invention directly targets blood vessels, the heterogeneity of the blood supply is not relevant. (Id.) Since in the method of the invention it is not required that the antibody extravasate into the tumor in the setting in which the vasculature itself is targeted, elevated interstitial pressure is not relevant. (Id.) And, of course, since again the method of the invention targets the blood vessels themselves and does not require antibody extravasation, large transport distances also is not relevant.(Id.)

**2. Data**

a. *In Vitro* Targeting Data

In addition to data presented in the application, further *in vitro* data demonstrating effective targeting (as well as ablation) of tumor cells using the antibodies of Applicant's invention is

presented in Yang et al., AACR Abstract #2996 (1998) and a poster presentation presented by Ballangrud et al. **(Bander Declaration #2, ¶11).** These data are generated using LNCaP spheroids which are LNCaP cells that grow *in vitro* as tumor masses several hundreds of microns in diameter, rather than in a monolayer. These spheroid masses recapitulate an *in vivo* tumor mass to an extent and demonstrate the ability of the antibodies of Applicant's invention to penetrate into tumor masses. Indeed, these spheroids are substantially larger that the typical prostate cancer metastasis. (Id.) As shown in the data, the antibodies of Applicant's invention, conjugated with a florescein marker or isotope, are observed by confocal microscopy to penetrate into these tumor masses. (Id.) Beyond this penetration, use of the radioisotope antibody conjugate, shows that these relatively large tumor spheroids can be effectively destroyed or killed. (Id.) Also included in Exhibit B to the **Bander Declaration #2** is a series of graphs and photos which examine the volume of multiple spheroids over time, treated with and without dexamethasone and/or $^{90}$Ytrium labeled specific (J591) or nonspecific (HuM195) antibody. (Id.)

b. *In Vivo* Targeting Data

In addition to the *in vitro* data mentioned above, *in vivo* animal data which demonstrates the ability of the antibodies of Applicant's invention to target tumor sites is appended in Exhibit C of the **Bander Declaration #2**. These 2 graphs examine Applicant's antibodies J591 and J415, as well as 7E11, conjugated to two different isotopes and demonstrate by quantitative analysis, that there is selective and specific uptake of radiolabeled antibody by PSMA–expressing tissues in an animal model (i.e. tumor xenografts). **(Bander Declaration #2, ¶12.)** More specifically, these graphs demonstrate that, over the 6-8 day period of observation, the relative amount of antibody in the tumor as compared to either blood or muscle continues to increase. (Id.)

Not shown on these graphs is that an irrelevant antibody (B1) showed significantly lower tumor to non-tumor ratios than those found with these antibodies specific for PSMA. (Id.)

Clinical *in vivo* data demonstrating targeting of the antibodies of Applicant's invention to a non-prostate cancer in a human patient is appended as Exhibit D of the **Bander Declaration #2**. The subject patient had both hormone-refractory prostate cancer and biopsy-proven colon cancer that had spread (metastasized) to the liver. **(Bander Declaration #2, ¶13.)** The two photographs represent the patient's CAT scan and the patient's antibody scan. (Id.) The CAT scan shows sequential slices through the liver demonstrating the mass in the right lobe of the liver. (Id.) As shown in the antibody scan of this patient following administration of a radiolabeled antibody of Applicant's invention, there is intense uptake of radiolabel and therefore significant signal in the vasculature of the same liver metastasis, indicating antibody localization to this non-prostate cancer. (Id.)

c. The Antibodies of the Invention Effectively Ablate or Kill Tumor Tissue

The Examiner also notes at page 7 and 8 of the Office Action that "the demonstration of *in vitro* binding to tissue samples provides insufficient objective evidence that the instant antibody-toxins are predictively effective in ablating or killing cancer cells in the *in vivo* clinical situation, based on *in vitro* binding to cells," and that "there is no indication that binding of the J591 antibody to live LNCaP cells had any effect on their viability." As noted above, the antibodies of Applicant's invention have been shown to target tumor cells *in vivo* in both an animal model and the clinical setting. Moreover, as also discussed above, *in vitro* data using LNCaP spheroids demonstrates the ability of the antibodies of Applicant's invention to kill LNCaP cells growing in a tumor mass.

i. Further *In Vitro* Ablation/Killing Data

Data from an additional *in vitro* experiment appended in Exhibit E of **the Bander Declaration #2** demonstrate that the J591 antibody (both humanized and mouse) mediates antibody dependent cellular cytotoxicity (ADCC). (**Bander Declaration #2, ¶15.**) That is, human lymphocytes and anti-PSMA antibody will induce a lysis of human prostate cancer cells. (Id.) Controls in the studies consist of no antibody and no effector cells, and a humanized and mouse versions of an anti-leukemia ("irrelevant") antibody plus cells. (Id.) This is yet another mechanism by which the antibodies of the present invention demonstrate their cytotoxicity. (Id.) And as explained below, the antibodies of Applicant's invention are effective in killing tumor cells *in vivo* in both an animal model and the clinical setting.

ii. *In Vivo* Ablation/Killing Data

The data appended in Exhibit F of the **Bander Declaration #2** shows that the use of a radiolabeled ($^{213}$Bismuth) antibody of Applicant's invention can delay and/or prevent tumor growth *in vivo* in an animal model. (**Bander Declaration #2, ¶16.**) In this animal study, the animals were inoculated with a human prostate xenograft of LNCaP cells and several days later were treated with $^{213}$Bismuth conjugated J591 antibody. (Id.) Two control groups were studied. One group received no treatment whatsoever and the second group received $^{213}$Bismuth conjugated to an irrelevant antibody which targets human leukemia cells but not prostate cancer cells. There were 6 animals per treatment group. (Id.) Figure 2 is the serum PSA (a prostate cancer antigen detectable in the serum of individuals with prostate cancer and an indicator of the presence/progression of prostate cancer) data of the mice shown in Figure 1. (Id.) In brief, the data shows that on day 51, the PSA levels in these mice is substantially higher in the two control groups than in the groups given anti-PSMA antibody conjugate. (Id.) This study demonstrates that there was a significant delay in the



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development of tumor growth in the anti-PSMA treated animals and half of those animals never developed detectable tumors. (Id.)

The first results of Applicant's recent experiments using antibody J591 conjugate in mice having large LNCaP xenograft tumors of approximately 1 cm in diameter, (this represents 5% of the animal's body weight) have shown similar xenograft killing effects. (**Bander Declaration #2, ¶17.**) Exhibit G of the **Bander Declaration #2** includes data from a number of different studies. Firstly, data labeled "G1", are results of a large series of animals treated with $^{131}$Iodine-muJ591 (mouse J591) at different doses (100mCi or 300 mCi) and different routes of administration (intraperitoneal and intravenous). (Id.) The animals in these studies got a single treatment dose on day 0 approximately 10-14 days post tumor implantation, when tumors had reached approximately 1 cm in diameter. Control animals received a single injection. Each line represents a growth curve for an individual tumor. (Id.) Exhibit "G2a" shows a point to point tracing of the average size of tumors in a group of animals (3-5 animals per group) treated with saline (PBS), J591 alone, J591 conjugated to a cytotoxic drug or animals treated with J591 conjugated to a different cytotoxic. (Id.) The same data appears in exhibit "Gb2" except that the curves are now "fitted" by a computer program. "Gb2" also indicates the number of animals in each group. (Id.) Exhibit "G2c" shows a plot of the weight of the animals in the different treatment groups showing that there was not significant adverse on the animals weight due to the treatment. However, the control (PBS) animals suffered the most with respect to weight as they became increasingly cachectic due to the increasing size of their tumors. (Id.) Exhibit "G3a" and "G3b" are graphs which show that the cytotoxic conjugates can effectively kill LNCaP cells *in vitro*. (Id.)

The data appended in Exhibit H of the **Bander Declaration #2** show that the use of radiolabeled antibodies and "naked" antibodies of Applicant's invention in a human patient are

effective at both imaging/localization to non-prostate cancer and resulting in a measurable shrinkage of tumor. More specifically, this patient received humanized J591 antibody. The first 6 doses of antibody included a combination of "naked" antibody and antibody labeled with a trace amount of $I^{131}$ which was for diagnostic purposes only and not intended as a therapeutic dose. Thereafter, this patient received 3 doses of purely "naked" antibody. The four photographs in Exhibit H are from the CAT scan and indicate nodal involvement in the neck, mediastinum, retroperitoneum and retrocrural area and pelvis. An additional photo is the patient's bone scan. Also included is a PET scan which demonstrates uptake of radiolabeled antibody in most of the areas shown on the CAT scan. The photograph labeled "H1" is the planar scan of the patient's antibody study which demonstrates uptake in the left neck node, mediastinum and retroperitoneum as well as pelvic nodes. This can also be seen on the SPECT study labeled "H2" where uptake in the neck node, mediastinum as well as the right shoulder (consistent with the increased uptake in the right shoulder on the bone scan). As of the date of the Bander Declaration #2, this patient had a 25% shrinkage of his measurable left neck mass and a 50% decline in his PSA.

In view of the foregoing, Applicant submits that this case is in condition for allowance and such allowance is earnestly solicited.

Respectfully submitted,

LYON & LYON LLP

Dated: March 12, 2001

By: _____
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68. (Amended) A method of prophylactic treatment of prostate cancer comprising:

providing an isolated antibody or antigen binding portion thereof which, when contacted with an extracellular domain of prostate specific membrane antigen present as an integral membrane protein in a living cell, binds to the extracellular domain of prostate specific membrane antigen; and

contacting prostate cells with the isolated antibody or antigen binding portion thereof under conditions to permit both binding of the isolated antibody or antigen binding portion thereof to the prostate cells and ablating or killing of the cells to prevent or delay development or progression of prostate cancer.

## VERSION WITH MARKINGS TO SHOW CHANGES MADE

For the paragraph beginning on page 12, line 29 (brackets indicate deletions and double dashes indicate insertions):

Figure 8 is a comparison of the heavy chain of monoclonal antibody J591 with the consensus sequence for Mouse Heavy Chains Subgroup IIA --(designated SEQ ID No. 20) --.

For the paragraph beginning on page 13, line 5:

Figure 11 is a comparison of the kappa light chain of monoclonal antibody J591 with the consensus sequence for Mouse Kappa Chains Subgroup V --(designated SEQ ID No. 21) --.

For the paragraph beginning on page 42, line 10:

The J591 VH is in Mouse Heavy Chains Subgroup IIA --(designated SEQ ID No. 20)-- (Kabat et al., Sequences of Proteins of Immunological Interest, U.S. Department of Health and Human Services (1991) ("Kabat"), which is hereby incorporated by reference). The sequence of J591 VH is compared to the consensus sequence for this subgroup in Figure 8.

For the paragraph beginning on page 45, line 4:

J591 VK is in the Mouse Kappa Chains Subgroup V --(designated SEQ ID No. 21)-- (Kabat, which is hereby incorporated by reference). The sequence of J591 VK corresponding to the ten identical clones is compared to the consensus sequence for the subgroup in Figure 11.

## CLAIM SHEETS MARKED UP TO SHOW CHANGES

1. (Twice amended) A method of ablating or killing normal, benign hyperplastic, and cancerous prostate epithelial cells comprising:

providing an isolated antibody or antigen binding portion thereof [a biological agent] which binds to an extracellular domain of prostate specific membrane antigen present as an integral membrane protein in a living cell, and

contacting said cells with the isolated antibody or antigen binding portion thereof [biological agent] under conditions effective to permit both binding of the isolated antibody of antigen binding portion thereof [biological agent] to the extracellular domain of the prostate specific membrane antigen and ablating or killing of said cells.

2. (Amended) A method according to claim 1, wherein the isolated antibody or antigen binding portion thereof [biological agent] is internalized with the prostate specific membrane antigen.

4. (Amended) A method according to claim 1, wherein said contacting is carried out in a living mammal and comprises:

administering the isolated antibody or antigen binding portion thereof [biological agent] to the mammal under conditions effective to permit both binding of the biological agent to the extracellular domain of the prostate specific membrane antigen and killing of said cells.

4 5. (Amended) A method according to claim 3, wherein the isolated antibody or antigen binding portion thereof [biological agent] is internalized with the prostate specific membrane antigen.

5 6. (Amended) A method according to claim 1 [2], wherein [an antibody is used in carrying out said method,] the antibody is [being] selected from the group consisting of a monoclonal antibody and a polyclonal antibody.

1 10. (Amended) A method according to claim 1 [2], wherein an antigen binding portion of an antibody is used in carrying out said method, the binding portion being selected from the group consisting of an Fab fragment, an F(ab')2 fragment, and an Fv fragment.

10 12. (Amended) A method according to claim 1, wherein the isolated antibody or antigen binding portion thereof [biological agent] is bound to a substance effective to kill or ablate said cells upon binding of the isolated antibody or antigen binding portion thereof [biological agent] to the extracellular domain of the prostate specific membrane antigen of said cells.

13 15. (Amended) A method according to claim 1 [2], wherein the antibody is effective to initiate an endogenous host immune function.

14 16. (Amended) A method according to claim 13 15, wherein the endogenous host immune function is complement-mediated cellular cytotoxicity [cytoxicity].

15 17. (Amended) A method according to claim 13, wherein the endogenous host immune function is antibody-dependent cellular cytotoxicity [cytoxicity].

16 18. (Amended) A method according to claim 1, wherein the isolated antibody or antigen binding portion thereof [ biological agent] is in a composition further comprising a physiologically acceptable carrier, excipient, or stabilizer.

17 19. (Amended) A method according to claim 1, wherein the isolated antibody or antigen binding portion thereof [ biological agent] is in a composition further comprising a pharmaceutically acceptable carrier, excipient, or stabilizer.

68. [80.] (Amended) A method of prophylactic treatment of prostate cancer comprising:

providing an isolated antibody or antigen binding portion thereof [a biological agent] which, when contacted with an extracellular domain of prostate specific membrane antigen present as an integral membrane protein in a living cell, binds to the extracellular domain of prostate specific membrane antigen; and

contacting prostate cells with the isolated antibody or antigen binding portion thereof [biological agent] under conditions to permit both binding of the [biological prostate cells with the biological agent under conditions to permit both the binding of the ] isolated antibody or antigen binding portion thereof [biological agent] to the prostate cells and ablating or killing of the cells to prevent or delay development or progression of prostate cancer.

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re the Application of:

**Neil H. Bander**

**Serial No.:** 08/838,682

**Filed:** April 9, 1997

**For:** TREATMENT AND DIAGNOSIS OF PROSTATE CANCER

**Group Art Unit:** 1642

**Examiner:** Yvonne Eyler

## DECLARATION OF NEIL H. BANDER UNDER 37 CFR § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, NEIL H. BANDER, pursuant to 37 C.F.R. § 1.132, declare:

1. I hold a B.A. degree in Biology from Johns Hopkins University and a M.D. degree from the University of Connecticut Medical School.

2. I am a Professor of Urology, Weill Medical College of Cornell University where I am also the Bernard and Josephine Chaus Chair in Urologic Oncology and Surgical Director of Urologic Oncology.

3. I am an Attending Surgeon, Department of Urology, New York Presbyterian Hospital-Cornell University Medical Center.

CERTIFICATE OF MAILING
(37 C.F.R. §1.10)

I hereby certify that this paper (along with any referred to as being attached or enclosed) is being deposited with the United States Postal Service on the date shown below with sufficient postage as 'Express Mail Post Office To Addressee' in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

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Felicia Reyes
Name of Person Mailing Paper

Felicia Reyes
Signature of Person Mailing Paper

4. I am also an Assistant Member at Memorial Sloan-Kettering Cancer Center, a Clinical Assistant Surgeon in the Urology Service and Clinical Immunology Service at Memorial Hospital, a Research Associate at Sloan-Kettering Institute for Cancer Research, and an Assistant Member of the Ludwig Institute for Cancer Research at Memorial Sloan-Kettering Cancer Center.

5. As explained more fully in my attached *Curriculum vitae,* I have authored numerous publications in the field of Urologic Oncology. (**Exhibit A.**)

6. I am the sole inventor of the above-identified patent application.

7. I have reviewed the Office Action mailed from the United States Patent Office on April 27, 1999, regarding the above-identified patent application. In this paper, the Examiner states at page 3 that "it is unclear if a cell line which produces an antibody...can be reproducibly isolated without undue experimentation." To the contrary, the procedures for producing the antibodies of my invention are fully described in the application. In particular, the four antibodies described in the application (i.e. E99, J415, J533 and J591) were prepared as set forth in Example 3. In fact, I used this same approach more than once to generate the four different antibodies. Antibody E99 is derived from a completely different immunization/fusion experiment than the remaining antibodies denoted by the "J" prefix, i.e. J415, J533 and J591.

8. I have reviewed Murphy et. al., "Measurement of Prostate-Specific Membrane Antigen in the Serum with a New Antibody," The Prostate 28: 266-71(I 996)("Murphy") and believe the work it describes is highly distinguishable from my present invention.

9. PSMA is a highly glycosylated integral membrane protein in a living cell and as such, it has specific 3-dimensional folding characteristics which results in exposure of epitopes, or antigen binding sites, which may not be sequential as well as the masking of certain peptides

by carbohydrate residues. Murphy utilizes the known linear peptide sequence of PSMA protein to derive and synthesize specific peptides to be used for immunization. This is a key difference from the approach I used to make the present invention. In particular, Murphy's selection of peptides is artificial and arbitrary and it cannot predictably and reliably select peptides which are, in fact, immunogenic. Furthermore, the peptide approach does not take into account the fact that PSMA is a highly glycosylated molecule and some or all of the selected peptides may be masked by carbohydrate residues of the native molecule. Of at least equal importance is that a selected peptide, because it exists somewhat in isolation and out of the context of the remainder of the molecule or, for that matter, neighboring molecules, does not possess the appropriate secondary, tertiary or quartenary structure in order to predictably and reliably generate antibodies capable of recognizing the native glycoprotein in its physiological state. Conversely, my method, by using viable, unmodified LNCaP cells, presents the native PSMA glycoprotein to the immunized animal's immune system in a form whereby generated antibodies do recognize the native glycoprotein as it exists as an integral membrane protein of a living cell.

10. Murphy describes a monoclonal antibody (called 3F5.4G, referred to herein as "3F5") produced by immunization with an 8 amino acid peptide derived from the published sequence of PSMA. This peptide resides at positions 716-723 towards the carboxy-terminus (external domain) of the protein. There is no evidence that the 3F5 antibody binds to an extracellular domain of PSMA present as an integral membrane protein of a living cell. Moreover, the data presented indicate that 3F5 may not bind to the same antigen as 7E11.C5 ("7E11").

11. In Figure I (pg. 268), Murphy provides a Western Blot with 2 lanes, 1 representing 7E11 and the other representing 3F5. These lanes represent the identical LNCaP

lysate probed with the respective antibodies. The 7Ell lane demonstrates a broad band of reactivity at a level indicated in the figure to represent PSMA. The 3F5 lane demonstrates a much narrower band purported to be at the same level. No molecular weight standards are provided to allow for comparability of the lanes or comparability of the molecular weights of the identified bands. Furthermore, the putative PSMA bands in each lane have entirely different characteristics, with the 7E11 band being much broader than the 3F5 band. The figure legend itself notes quite equivocally "that 3F5.4G6 recognizes a protein of $M_r$ 120kDa, which is similar, if not identical, to the protein recognized by 7E11.C5."

12. In Figure 3 (pg. 270), Murphy shows an immunoprecipitate-Western-Blot where the LNCaP lysate is initially immunoprecipitated with 7E11 and then probed with either 7E11 or 3F5. If 3F5 identifies PSMA, lanes 5 and 6, which are blotted with 3F5, should closely mirror lanes 1 and 2 which are probed with 7E11. However, lanes 5 and 6 show only a very weak band of reactivity and are not even at the same level as the 7E11 bands. Lanes 3 and 7, which represent LNCaP lysate immunoprecipitated with 7E11 and subsequently blotted with 7E11 (lane 3) or 3F5 (lane 7), should also provide specific comparability of the respective immunoreactivities. However, these 2 lanes blot so much background or nonspecific immunoreactivity that they are useless in identifying these 2 antibodies as binding the same protein. Lanes 4 and 8 should show only a single band representing "PSM-prime" ("PSM'"); however, lane 8 shows 2 bands, 1 of which is neither explained nor commented on by Murphy. Figure 3b represents LNCaP lysate immunoprecipitated with 3F5 and probed in a Western Blot with antibody 7E11. Again, the 2 lanes should show identical immunoreactivities but demonstrate different patterns, further suggesting that these 2 antibodies do not recognize the same target antigen.

13. Figure 4 of Murphy provides Western Blot analyses of serum from a prostate cancer patient. Murphy indicates that the 7E11 antibody reacts with a band present in serum at a molecular weight close to that of PSMA. However, other investigators including Troyer et al. (references 5 and 6 in the Murphy paper) have published data indicating that the immunoreactivity of 7E11 on Western Blots of serum does not, in fact, react with PSMA. Troyer et al. demonstrated that one could not compete for the 7E11 binding of this serum band using a peptide containing amino acids 1-19 of PSMA which incorporates the previously mapped 7E11 epitope of PSMA. Others have also not been able to confirm that the legitimate PSMA molecule is present in serum. I have found the same cross-reaction of 7E11 with a non-PSMA molecule in serum described in Troyer et al. It is believed that the 7E11 molecule cross-reacts with a similar protein found in serum but which is different from that found associated with the cell membrane of prostate cancer cells and which would explain why the 7E11 epitope does not compete for this binding. Lanes 3 and 4 in Figure 4 of Murphy demonstrate that the 3F5 antibody identifies the same serum band as 7E11.

14. In conclusion, Murphy immunizes with a peptide, failing to take into consideration the heavily glycosylated nature and 3-dimensional structure of the native protein. He produces an IgM antibody. These antibodies are typically of low affinity and that appears to be the case with this particular antibody based on the intensity of the blot. The 3F5 antibody reacts with a molecule present in serum "identical" to the serum molecule identified by 7E11.C5. Most investigators believe that this molecule is different from prostate epithelial cell membrane-associated PSMA and that this is merely a molecule which cross-reacts with PSMA. Murphy provides no evidence, nor for that matter does he even claim, that his 3F5 antibody binds to LNCaP cells or, for that matter, that it binds to viable LNCaP cells. This antibody

appears to be entirely different from the 4 antibodies of my present invention, none of which react with a protein found in serum to an extent to give rise to background noise which interferes with *in vivo* imaging and all of which can be found to bind viable LNCaP cells. On this point, an antibody (such as 7E11 or 3F5) which binds a serum molecule would be different from, and less desirable than, an antibody which does not bind a serum molecule, if the purpose of that antibody were *in vivo* targeting of tumor. Antibody binding to a serum constituent would be 'decoyed' by the serum molecule and the antibody would never find its cellular target. The complexing of antibody in serum by the circulating molecule would further add to 'background', further diminishing *in vivo* diagnostic or therapeutic capability. In addition, the complexing of serum antigen to such antibodies bound to cytotoxic agents would be harmful to other organs.

15. It is incorrect that extracellular antigens are all internalized. Some (and probably most) such antigens are anchored in the cell membrane and do not internalize. Not all (nor even many) extracellular antigens can be induced to be internalized by antibody binding. At the time that the antibodies of my present invention were developed, those skilled in this area, as demonstrated by Israeli et al., Cancer Res. 53:227-230 (1993) and Heston, Urology 49[supp 13A]:104-112 (1997) (see pg. 106), believed that PSMA lacked an internalization sequence and therefore was not internalized.

16. Coleman, et. al., Fundamental Immunology (1989)("Coleman") is not applicable to my present invention. This reference describes the process of "capping" and "pinocytosis" and relates to cells of hematopoietic lineage not of epithelial origin such as prostate cancer. One cannot freely extrapolate observations on hematopoietic cells to epithelial cells. Indeed, the specific mechanism of internalization described by Coleman is entirely different from the internalization mechanism with respect to antibody binding to the extracellular domain of

PSMA. In particular, Coleman points out the requirement for bivalent or multivalent antibodies, indicating that monovalent antibodies are incapable of inducing internalization. By contrast, I have found that even monovalent antibodies like those of my present invention are internalized. Lastly, Coleman describes cells normally losing surface determinants in the internalization process with later re-expression of these determinants. I have never found loss of the surface expression of the surface determinants and, indeed, find that regardless of the presence or absence of antibody, the extracellular domain of PSMA is continuously present.

17. I have reviewed Israeli et al., Cancer Res. 53:227-230 (1993) ("Israeli") and believe the work it describes is highly distinguishable from my present invention. Indeed, Israeli teaches one away from my invention by indicating that synthetic peptides should be used to make anti-PSMA antibodies. In using synthetic amino acid sequences of PSMA as an immunogen to develop antibodies to PSMA, one cannot be certain how well exposed such a peptide is on a living cell, nor how immunogenic it is. Furthermore, this approach does not take into consideration the 3 dimensional folding of the native PSMA molecule, nor its glycosylation or other post-translational modifications and other characteristics which are of significant importance in an antibody response to a native antigen. Also, an immunogenic peptide may not be useful in the context of a heavily glycosylated molecule. Peptides, therefore, cannot effectively substitute for the natural tertiary and quarterary structure of a protein in a physiological situation. I am not aware of any antibodies that have been made using such a synthetic peptide of PSMA that can successfully bind to viable prostate cancer cells. I am however, aware of researchers who have tried, yet failed, at such an approach.

18. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that

these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

DATE: 7/19/99

NEIL H. BANDER

# CURRICULUM VITAE

**Name**: Neil Harrison Bander

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**Education**:

Johns Hopkins University
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Intern,1974-75; Junior Resident, 1975-76; Resident, General Surgery, 1976-77, New York University-Bellevue Medical Center

Resident, Urology, 1977-78; Senior Resident, Urology, 1978-79; Chief Resident, Urology, 1979-80, University of Connecticut Medical Center

Research Fellow, Immunology, Sloan-Kettering Institute for Cancer Research, 1980-83

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**Academic, Hospital and Research Positions and Appointments**:

Professor of Urology, Weill Medical College of Cornell University; Incumbent, Bernard and Josephine Chaus Chair in Urologic Oncology; Surgical Director of Urologic Oncology

Attending Surgeon, Department of Urology, New York Presbyterian Hospital-Cornell University Medical Center

Research Associate, Memorial Sloan-Kettering Cancer Center, 1984-85; Assistant Member, 1985-present

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Fellow, American College of Surgery
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Member, Medical Advisory Board, National Kidney Cancer Association
Cancer and Leukemia Group B (CALGB), GU Committee, 1989-94
Organizing Committee, IXth International Conference on Monoclonal Antibody Immunoconjugates, 1993-94
Chair, Medical Research Committee, New York Section, American Urological Association, 1994-95
Member, American Association of Clinical Urologists, Inc.

**Honors and Awards**:

American Cancer Society Pre-doctoral Research Fellowship, Dana-Farber Cancer Center/Harvard Medical School (mentor: Sidney Farber, M.D.), 1969-70
Mosby Scholarship Award, University of Connecticut Medical School, 1973-74
NIH Immunobiology Fellowship, Memorial Sloan-Kettering Cancer Center (mentor: Lloyd J. Old, M.D.), 1980-83
New York Academy of Medicine, Ferdinand C. Valentine Fellow in Urology (mentor: Willet F. Whitmore, Jr., M.D., Memorial Sloan-Kettering Cancer Center, 1982-83
Society of Surgical Oncology Ewing Research Award, 1983
Deutsche Gesellschaft fur Urologie (German Urological Association) Prize for Experimental Research, 1989
Visiting Lecturer, Japanese Urological Association, 1992
Association for the Cure of Prostate Cancer (CaP Cure) Award, 1993, 1994, 1995, 1996, 1997,1998

**Other Activities**:

Member, Cancer Clinical Investigation Review Committee (CCIRC) - Ad Hoc Committee on Urologic Oncology Cooperative Groups, National Institutes of Health, 1988
Site Review Committee, National Cancer Institute, 1988
Cancer Care & Research Committee, New York Hospital-Cornell Medical Center, 1994-present
Advisory Board, Prostate Cancer Infolink, 1995-present
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Human Genetics and Gene Therapy Working Group, New York Hospital-Cornell Medical Center, 1996-present
Review Committee, Cancer Research Institute's Prostate Cancer Initiative Clinical Trials Program, 1996, 1997
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CaP CURE Grant Review Committee, 1997
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PATENT
242/027

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

| | |
|---|---|
| In re the Application of: | **Group Art Unit:** 1642 |
| **Neil H. Bander** | **Examiner:** Yvonne Eyler |
| **Serial No.:** 08/895,914 | |
| **Filed:** July 17, 1997 | |
| **For:** TREATMENT AND DIAGNOSIS OF CANCER | |

## DECLARATION OF NEIL H. BANDER UNDER 37 CFR § 1.132

Box CPA
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, NEIL H. BANDER, pursuant to 37 C.F.R. § 1.132, declare as follows:

1. I hold a B.A. degree in Biology from Johns Hopkins University and a M.D. degree from the University of Connecticut Medical School.

2. I am a Professor of Urology, Weill Medical College of Cornell University where I am also the Bernard and Josephine Chaus Chair in Urologic Oncology and Surgical Director of Urologic Oncology.

3. I am an Attending Surgeon, Department of Urology, New York Presbyterian Hospital-Cornell University Medical Center.

CERTIFICATE OF MAILING
(37 C.F.R. §1.10)

I hereby certify that this paper (along with any referred to as being attached or enclosed) is being deposited with the United States Postal Service on the date shown below with sufficient postage as 'Express Mail Post Office To Addressee' in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

EL199138739US
Express Mail Label No.

July 20, 1999
Date of Deposit

Felicia Reyes
Name of Person Mailing Paper

Felicia Reyes
Signature of Person Mailing Paper

LA-86437.1

4. I am also an Assistant Member at Memorial Sloan-Kettering Cancer Center, a Clinical Assistant Surgeon in the Urology Service and Clinical Immunology Service at Memorial Hospital, a Research Associate at Sloan-Kettering Institute for Cancer Research, and an Assistant Member of the Ludwig Institute for Cancer Research at Memorial Sloan-Kettering Cancer Center.

5. As explained more fully in my attached *Curriculum vitae,* I have authored numerous publications in the field of Urologic Oncology. **(Exhibit A.)**

6. I am the sole inventor of the above-identified patent application.

7. I have reviewed the Office Action mailed from the United States Patent Office on January 21, 1999 regarding the above-identified patent application. In this paper, the Examiner states at page 4 that "the specification does not provide sufficient guidance and evidence to enable one of skill in the art to predictably obtain agents or antibodies that bind to the extracellular domain of PSMA and target tumor vasculature." To the contrary, the procedures for producing the antibodies of my invention are fully described in the application. In particular, the four antibodies described in the application (i.e. E99, J415, J533 and J591) were prepared as set forth in Example 3. In fact, I used this same approach more than once to generate the four different antibodies. Antibody E99 is derived from a completely different immunization/fusion experiment than the remaining antibodies denoted by the "J" prefix, i.e. J415, J533 and J591.

8. In addition, at page 5 of the January 21, 1999 Office Action, the Examiner states that "the specification provides evidence of the ability of the antibodies to target to tissues *in vitro* for detection but provides insufficient objective evidence that antibodies to PSMA extracellular domain or the antibodies E99, J415, J533 and J591 effectively target to tumor vasculature *in vivo*." The Examiner cites Jain, R.K. (Cancer and Metastasis Reviews, 9:753-266,

1990) as teaching "that the efficacy in cancer treatment of novel therapeutic agents such as monoclonal antibodies, cytokines and effectors cells has been limited by their inability to reach their target *in vivo* in adequate quantities." Several physiological factors "responsible for the poor localization" are set forth by the Examiner including "(I) heterogeneous blood supply, (ii) elevated interstitial pressure which lowers fluid extravasation, and (iii) large transport distances in the interstitium." As I detail below, these factors are not relevant in the approach or clinical setting of my invention and I provide additional *in vitro* and *in vivo* data as evidence that the antibodies of my invention do effectively target both prostate tumors and non-prostate tumor vasculature.

9. The factors recited by the Examiner are not relevant to the use of the antibodies of my invention for the detection and treatment of prostate cancer. First, metastatic prostate cancer predominantly involves the bone marrow and lymph nodes. These sites are, by definition, highly vascularized. In addition, prostate cancer is unlike many other solid tumors in that its metastatic sites are small volume sites measured in microns or millimeters. For this reason, as well as because of their presence in bone marrow and lymph nodes, these sites are very well supplied with antibody given the high levels of circulating antibody in the vascular compartment. Large transport distances, as a result, are not operative in this situation. Similarly, interstitial pressure in these small volume sites within bone marrow and lymph node is also not operative. Moreover, primary prostate cancers are also relatively small and multifocal and the factors recited by the Examiner are likewise not relevant.

10. With respect to targeting the PSMA molecule on vascular endothelial cells with the antibodies of my invention, the factors cited by the Examiner similarly are not operative. Since blood supply is necessary to allow tumor nutrition and growth, and since we are directly

targeting blood vessels, the heterogeneity of blood supply is not relevant. Since we do not require antibody to extravasate into the tumor in the setting in which we are targeting the vasculature itself, elevated interstitial pressure is not relevant. And, of course, since again we are targeting the blood vessels themselves and do not require antibody extravasation, the issues of large transport distances also is not relevant.

11. In addition to data presented in the application, further *in vitro* data demonstrating effective targeting (as well as ablation) of tumor cells using the antibodies of my invention is presented in Yang et al., AACR Abstract #2996 (1998) and a poster presentation presented by Ballangrud et al., attached as **Exhibit B.** These data are generated using LNCaP spheroids which are LNCaP cells that grow *in vitro* as tumor masses several hundreds of microns in diameter, rather than in a monolayer. These spheroid masses recapitulate an *in vivo* tumor mass to an extent and demonstrate the ability of the antibodies of my invention to penetrate into tumor masses. Indeed, these spheroids are substantially larger than the typical prostate cancer metastasis. As shown in the data, the antibodies of my invention conjugated with a florescein marker or isotope, are observed by confocal microscopy to penetrate into these tumor masses. Beyond this penetration, use of the radioisotope antibody conjugate, shows that these relatively large tumor spheroids can be effectively destroyed or killed. Also included in Exhibit B is a series of graphs and photos which examine the volume of multiple spheroids over time, treated with and without dexamethasone and/or $^{90}$Ytrium labeled specific (J591) or nonspecific (HuM195) antibody.

12. In addition to the *in vitro* data mentioned above, *in vivo* animal data which demonstrates the ability of the antibodies of my invention to target tumor sites is appended in **Exhibit C.** These 2 graphs examine my antibodies J591 and J415, as well as 7E11, conjugated

to two different isotopes and demonstrate by quantitative analysis, that there is selective and specific uptake of radiolabeled antibody by PSMA–expressing tissues in an animal model (i.e. tumor xenografts). More specifically, these graphs demonstrate that, over the 6-8 day period of observation, the relative amount of antibody in the tumor as compared to either blood or muscle continues to increase. Not shown on these graphs is that an irrelevant antibody (B1) showed significantly lower tumor to non-tumor ratios than those found with these antibodies specific for PSMA.

13. Clinical *in vivo* data demonstrating targeting of the antibodies of my invention to a non-prostate cancer in a human patient is appended as **Exhibit D**. This patient **[#6]** had both hormone-refractory prostate cancer and biopsy-proven colon cancer that had spread (metastasized) to the liver. The two photographs represent the patient's CAT scan and the patient's antibody scan. The CAT scan shows sequential slices through the liver demonstrating the mass in the right lobe of the liver. As shown in the antibody scan of this patient following administration of a radiolabeled antibody of my invention, there is intense uptake of radiolabel and therefore significant signal in the vasculature of the same liver metastasis, indicating antibody localization to this non-prostate cancer.

14. The Examiner also notes at page 6 of the Office Action that "the demonstration of *in vitro* binding to tissue samples provides insufficient objective evidence that the instant antibody-toxins are predictively effective in ablating or killing cancer cells in the *in vivo* clinical situation based on *in vitro* binding to cells," and that "there is no indication that binding of the J591 antibody to live LNCaP cell[s] had any effect on their viability." As noted above in paragraphs 11 and 12, the antibodies of my invention have been shown to target tumor cells *in vivo* in both an animal model and the clinical setting. Moreover, in paragraph 10, *in vitro* data

using LNCaP spheroids demonstrates the ability of the antibodies of my invention to kill LNCaP cells growing in a tumor mass.

15. Data from an additional *in vitro* experiment appended in **Exhibit E** demonstrate that the J591 antibody (both humanized and mouse) mediates antibody dependent cellular cytotoxicity (ADCC). That is, human lymphocytes and anti-PSMA antibody will induce a lysis of human prostate cancer cells. Controls in the studies consist of no antibody and no effector cells, and humanized and mouse versions of an anti-leukemia ("irrelevant") antibody plus cells. This is yet another mechanism by which the antibodies of the present invention demonstrate their cytotoxicity. And as explained below, we have shown the antibodies of my invention are effective in killing tumor cells *in vivo* in both an animal model and the clinical setting.

16. The data appended in **Exhibit F** shows that the use of a radiolabeled ($^{213}$Bismuth) antibody of my invention can delay and/or prevent tumor growth *in vivo* in an animal model. In this animal study, the animals were inoculated with a human prostate xenograft of LNCaP cells and several days later were treated with $^{213}$Bismuth conjugated J591 antibody. Two control groups were studied. One group received no treatment whatsoever and the second group received $^{213}$Bismuth conjugated to an irrelevant antibody which targets human leukemia cells but not prostate cancer cells. There were 6 animals per treatment group. Figure 2 is the serum PSA (a prostate cancer antigen detectable in the serum of individuals with prostate cancer and an indicator of the presence/progression of prostate cancer) data of the mice shown in Figure 1. In brief, the data shows that on day 51, the PSA levels in these mice is substantially higher in the two control groups than in the groups given anti-PSMA antibody conjugate. This study demonstrates that there was a significant delay in the development of tumor growth in the anti-PSMA treated animals and half of those animals never developed detectable tumors.

17. The first results of our recent experiments using antibody J591 conjugate in mice having large LNCaP xenograft tumors of approximately 1 cm in diameter, (this represents 5% of the animal's body weight) have shown similar xenograft killing effects. **Exhibit G** includes data from a number of different studies. Firstly, data labeled "G1", are results of a large series of animals treated with $^{131}$Iodine-muJ591 (mouse J591) at different doses (100mCi or 300 mCi) and different routes of administration (intraperitoneal and intravenous). The animals in these studies got a single treatment dose on day 0 approximately 10-14 days past tumor implantation, when tumors had reached approximately 1 cm in diameter. Control animals received a single injection. Each line represents a growth curve for an individual tumor. Exhibit "G2a" shows a point to point tracing of the average size of tumors in a group of animals (3-5 animals per group) treated with saline (PBS), J591 alone, J591 conjugated to a cytotoxin or animals treated with J591 conjugated to a different cytotoxin. The same data appears in exhibit "G2b" except that the curves are now "fitted" by a computer program. "G2b" also indicates the number of animals in each group. Exhibit "G2c" shows a plot of the weight of the animals in the different treatment groups showing that there was not significant adverse effect on the animals weight due to the treatment. However, the control (PBS) animals suffered the most with respect to weight as they became increasingly cachectic due to the increasing size of their tumors. Exhibit "G3a" and "G3b" are graphs which show that the cytotoxic conjugates can effectively kill LNCaP cells *in vitro*.

18. The data appended in **Exhibit H** show that the use of radiolabeled antibodies and "naked" antibodies of my invention in a human patient are effective at both imaging/localization to non-prostate cancer and resulting in a measurable shrinkage of tumor. More specifically, this patient received humanized J591 antibody. The first 6 doses of antibody included a combination

of "naked" antibody and antibody labeled with a trace amount of $I^{131}$ which was for diagnostic purposes only and not intended as a therapeutic dose. Thereafter, this patient received 3 doses of purely "naked" antibody. The four photographs in Exhibit H are from the CAT scan and indicate nodal involvement in the neck, mediastinum, retroperitoneum and retrocrural area and pelvis. An additional photo is the patient's bone scan. Also included is a PET scan which demonstrates uptake of radiolabeled antibody in most of the areas shown on the CAT scan. The photograph labeled "H1" is the planar scan of the patient's antibody study which demonstrates uptake in the left neck node, mediastinum and retroperitoneum as well as pelvic nodes. This can also be seen on the SPECT study labeled "H2" where uptake in the neck node, mediastinum as well as the right shoulder (consistent with the increased uptake in the right shoulder on the bone scan). This patient has, to date, had a 25% shrinkage of his measurable left neck mass and a 50% decline in his PSA.

19. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

DATE: 7/19/99

NEIL H. BANDER

# CURRICULUM VITAE

**Name**: Neil Harrison Bander

**Place of Birth**: Boston, MA

**Nationality**: U.S.A.

**Education**:

Johns Hopkins University
1969, B.A. (Biology)

University of Connecticut Medical School
1974, M.D.

**Postdoctoral Training**:

Intern,1974-75; Junior Resident, 1975-76; Resident, General Surgery, 1976-77, New York University-Bellevue Medical Center

Resident, Urology, 1977-78; Senior Resident, Urology, 1978-79; Chief Resident, Urology, 1979-80, University of Connecticut Medical Center

Research Fellow, Immunology, Sloan-Kettering Institute for Cancer Research, 1980-83

Fellow, Urologic Oncology, Memorial Hospital, 1982-83

**Academic, Hospital and Research Positions and Appointments**:

Professor of Urology, Weill Medical College of Cornell University; Incumbent, Bernard and Josephine Chaus Chair in Urologic Oncology; Surgical Director of Urologic Oncology

Attending Surgeon, Department of Urology, New York Presbyterian Hospital-Cornell University Medical Center

Research Associate, Memorial Sloan-Kettering Cancer Center, 1984-85; Assistant Member, 1985-present

Clinical Assistant Surgeon, Urology Service, Memorial Hospital, 1985-present; Clinical Assistant Surgeon, Clinical Immunology Service, 1985-present

Research Associate, Sloan-Kettering Institute for Cancer Research, 1983-present

Assistant Member, Ludwig Institute for Cancer Research
at Memorial Sloan-Kettering Cancer Center), 1992-present

**Licensed Physician**: State of New York (#126232)

**Board Certification**: Urology (1983)

**Scientific and Medical Societies**:

Fellow, American College of Surgery
American Urological Association
Association for Academic Surgery
Society of Urologic Oncology
American Society of Clinical Oncology
American Association of Cancer Research
Member, Medical Advisory Board, National Kidney Cancer Association
Cancer and Leukemia Group B (CALGB), GU Committee, 1989-94
Organizing Committee, IXth International Conference on Monoclonal Antibody Immunoconjugates, 1993-94
Chair, Medical Research Committee, New York Section, American Urological Association, 1994-95
Member, American Association of Clinical Urologists, Inc.

**Honors and Awards**:

American Cancer Society Pre-doctoral Research Fellowship, Dana-Farber Cancer Center/Harvard Medical School (mentor: Sidney Farber, M.D.), 1969-70
Mosby Scholarship Award, University of Connecticut Medical School, 1973-74
NIH Immunobiology Fellowship, Memorial Sloan-Kettering Cancer Center (mentor: Lloyd J. Old, M.D.), 1980-83
New York Academy of Medicine, Ferdinand C. Valentine Fellow in Urology (mentor: Willet F. Whitmore, Jr., M.D., Memorial Sloan-Kettering Cancer Center, 1982-83
Society of Surgical Oncology Ewing Research Award, 1983
Deutsche Gesellschaft fur Urologie (German Urological Association) Prize for Experimental Research, 1989
Visiting Lecturer, Japanese Urological Association, 1992
Association for the Cure of Prostate Cancer (CaP Cure) Award, 1993, 1994, 1995, 1996, 1997,1998

**Other Activities**:

Member, Cancer Clinical Investigation Review Committee (CCIRC) - Ad Hoc Committee on Urologic Oncology Cooperative Groups. National Institutes of Health, 1988
Site Review Committee, National Cancer Institute. 1988
Cancer Care & Research Committee, New York Hospital-Cornell Medical Center, 1994-present
Advisory Board, Prostate Cancer Infolink, 1995-present
Scientific Advisory Board, BZL Biologics, Inc., 1995-present
Human Genetics and Gene Therapy Working Group, New York Hospital-Cornell Medical Center, 1996-present
Review Committee, Cancer Research Institute's Prostate Cancer Initiative Clinical Trials Program, 1996, 1997
Reviewer, Ohio Cancer Research Associates, 1995, 1997
CaP CURE Grant Review Committee, 1997
Consultant/Science Advisory Board - Prostagen, Inc., 1997-
Reviewer, Department of Defense-Prostate Cancer Research Program, Immunology Grant Review Panel #1, January, 1998
Immunology Grant Review Panel #2, May,1998
Member, Urologic Oncology Branch's Tenure Track Urologic Surgeon Search Committee, National Cancer Institute, 1998
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**Dependents #**: Gregory Bander (08/27/85)
Evan Bander (11/10/88)

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produced 25.3% and 64.9% lysis of SKOV3 cells, respectively. The B1D2 × NM3E2 $scFv_2$ promoted cytotoxicity at concentrations as low as 100pg/ml. Thus, high affinity binding to tumor antigens increases bispecific $scFv_2$-mediated cytotoxicity, possibly because the slow off-rate of the higher affinity bispecific $scFv_2$ prolongs its cell surface retention. Additional bispecific constructs are currently being tested to further examine the role of affinity over a 10,000-fold range of $K_D$ values.

**#2993** **Antibody-TNF fusion constructs targeting HER2 can overcome HER2-mediated resistance to TNF.** Rosenblum, M., Parakh, C., Horn, S., and Cheung, L. *University of Texas—M.D. Anderson Cancer Center, Houston, TX 77030.*

Overexpression of the proto-oncogene HER2/NEU in breast cancer and certain other tumors appears to be a central mechanism which may be partly responsible for cellular progression of the neoplastic phenotype. Transfection studies with HER2/NEU results in reduced sensitivity to TNF's cytotoxic effects and reduced sensitivity to immune effector killing. The single-chain recombinant antibody sfv23 recognizes the cell-surface domain of HER2. The cDNA for this antibody was fused to the cDNA encoding human TNF and this fusion construct was cloned into a plasmid for expression in E. coli. The fusion protein was expressed as insoluble inclusion bodies and renatured after solubilization in 6M guanidine and purified by ion exchange chromatography. SDS-PAGE demonstrated a single band at the expected molecular weight (43 kDa). The fusion construct was tested for TNF activity against L-929 cells and found to have TNF activity (S.A. 420 nM). The construct was also tested by ELISA for binding against SKBR-3 (HER2 positive) cells. Cytotoxicity studies against SKBR-3 cells demonstrate that the sfv23/TNF fusion construct was 500 fold more active than free TNF and therefore apparently able to overcome the HER-2 mediated resistance to TNF. Further in vitro studies to examine the biological properties of this agent are ongoing.

**#2994** **Characterization of Anti-HER-2 Monoclonal Antibodies Which Inhibit the Growth of Breast Cancer Cell Lines.** Ilgen, A., Ghetie, M., Shen, G., Li, J., Uhr, J. and E. Vitetta. *Cancer Immunobiology Center, UT Southwestern Medical Center.*

HER-2, or c-erbB-2, is a member of the EGF receptor family. Overexpression of the wild type HER-2 protein, as is observed on numerous carcinomas, leads to hyperactivity of the kinase and confers a significant growth advantage on cells. Numerous groups have generated monoclonal antibodies (MAbs) against HER-2 which inhibit the growth of breast cancer cell lines. Our research had three goals: to understand the mechanisms by which anti-HER-2 MAbs inhibit the growth of breast cancer cells, how the physical properties of the MAbs related to their mechanism of inhibition, and how we can optimize the anti-tumor activity of these MAbs. We generated a panel of 113 Mabs against HER-2 in this manner, 12 of which inhibited the growth of a panel of HER-2-overexpressing breast cancer cells. To understand the mechanism of inhibition of growth, we determined whether the MAbs induced cell cycle arrest and/or apoptosis in treated cells. We found that each of these 12 MAbs signaled the cells to undergo varying degrees of apoptosis and/or cell cycle arrest and that the signaling capabilities of the Mabs correlated with both the extent of overexpression of HER-2 on the breast cancer cell lines and the affinity of the MAbs. Our next step will be to determine whether MAbs and immunotoxins against different epitopes on the HER-2 molecule are able to synergistically inhibit the growth of the breast cancer cells.

**#2995** **Anti-metastatic therapy of MDR human lung cancer with anti-P-glycoprotein antibody and monocyte chemoattractant protein-1 gene transduction in SCID mice.** Nokihara, H., Hanibuchi, M., Yanagawa, H., Shinohara, T., Fujiki, F., Nishinuraa, N., Parajuli, P., Tsuruo, T., and Sone, S. *Third Department of Internal Medicine, Tokushima University School of Medicine, Tokushima 770.Japan. and Institute of Molecular and Cellular Biosciences, The University of Tokyo, Tokyo 113, Japan.*

Distant metastasis is a critical problem in the therapy of human lung cancer. In this study, we investigated whether transduction of the monocyte chemoattractant protein-1 (MCP-1)-gene into multidrug-resistant (MDR) human lung cancer cells affected the inhibition of their metastases by the anti-P-glycoprotein (P-gp) monoclonal antibody MRK16. MDR human small cell lung cancer (SCLC), H69/VP cells, were transduced with human MCP-1-gene inserted into an expression vector (BCMGSNeo). MCP-1-gene transduction had no effect on the expression of P-gp, drug sensitivity to etoposide or the in vitro proliferation. In the metastatic model of NK-cell depleted SCID mice, the number of metastatic colonies of MCP-1-gene transduced H69/VP cells were similar to those of parent or mock-transduced cells. However, systemic treatment with MRK16 was more effective in inhibiting the metastasis of MCP-1-producing H69/VP than mock-transduced cells. These findings suggest that local production of MCP-1 in tumor site may increase the anti-P-gp antibody dependent cell-mediated cytotoxicity. This can be a useful immunological strategy to inhibit the metastasis of MDR human lung cancer cells expressing P-gp.

**#2996** **Alpha particle emitter therapy of micrometastases: $^{213}$Bi-J59 (anti-PSMA) treatment of LNCaP spheroids.** Yang, W-H., Ballangrud, A., McDevitt, M.R. Finn, R.A., Geerlings, M., Bander N., Scheinberg, D.A., Sgouros, G. *Memorial Sloan-Kettering Cancer Center, NY, NY 10021. Pharmatinium, Inc. Wilmington, DE 19801, Cornell University Medical Center, NY, 10021.*

Multicellular spheroids were used to investigate the feasibility of eradicating micrometastases with radiolabeled antibodies. Spheroids of LNCaP (LN3) cells were exposed to 2 concentrations of bismuth-213-labeled J591 antibody (50 and 100 $\mu$Ci) for 4 different incubation durations (15,30,60 min and 18 h). J591 targets an extracellular epitope of the prostate-specific membrane antigen (PSMA). $^{213}$Bi emits alphas of 60 to 90 $\mu$m range; it has a 45.6 min. half-life. 24 spheroids, of 100 to 200 $\mu$m diameter were used for each experiment. Growth curves were obtained for each spheroid up to day 70, post-incubation. $^{213}$Bi-HuM195 (anti-CD33), was used as a "hot" control. Spheroids exposed to 100 $\mu$Ci $^{213}$Bi-J591 for more than 15 min. did not re-grow; controls showed growth delay but re-grew exponentially following an 18 h incubation. The growth of spheroids exposed to 50 $\mu$Ci $^{213}$Bi-J591 was arrested only after an 18 h exposure; at days 33 and 68, median volume ratios of 400 and 7400 between hot control and specific antibody were obtained. The results demonstrate feasibility and efficacy in using antibodies labeled with alpha particle emitting radionuclides to target small cluster of tumor cells or micrometastases. The results obtained using this spheroid model may be used, in combination, with mathematical modeling to evaluate different treatment protocols against micrometastases and to optimize such a treatment approach.

**#2997** **A novel humanised antibody against Prostate Specific Membrane Antigen (PSMA) for *in vivo* targeting and therapy.** Hamilton, A., King, S., Liu, C., Moy, P., Bander, N., and Carr, F. *Biovation Ltd, Aberdeen AB24 3RY, U.K. Department of Urology, The New York Hospital-Cornell Medical Center, New York, NY 10021, Ludwig Institute for Cancer Research, New York Branch, New York, NY 10021.*

A murine monoclonal antibody (mAb), J591, is directed the against the extracellular domain of PSMA, an integral membrane protein of prostate carcinoma and of tumour vascular endothelium of a wide variety of cancers, but not normal endothelium. The mAb was "humanised" by a novel method involving specific deletion of human B and T cell epitopes. To remove B cell epitope surface exposed residues in the frameworks of the murine J591 heavy and light chain variable region ($V_H$ and $V_L$) sequences were substituted by the corresponding residues from selected human germ-line $V_H$ and $V_L$ sequences ("surface humanisation"). For detection and elimination of T cell epitopes, a database of human MHC class II binding peptides was searched for motifs present in the substituted $V_H$ and $V_L$ sequences and in addition a novel computer modelling approach termed "peptide threading" was applied. Motifs, unless also present in human germ-line antibody sequences, were deleted by substituting single amino acids, preserving the CDRs. The final sequences were re-checked for new MHC class II motifs. The designed $V_H$ and $V_L$ regions were constructed by mutagenesis of the murine $V_H$ and $V_L$. Human IgG1 or $\kappa$ constant regions were added and the composite genes transfected into NS0 cells to produce complete recombinant antibodies. These antibodies bound to PSMA (on LNCap cells) as efficiently as the original murine antibody, and are expected have little or no immunogenicity in man.

**#2998** **Serum TA90 immune complex correlates with recurrence following adjuvant immunotherapy for regional metastatic melanoma.** Hsueh, E.C., Gupta, R.K., Yee, R., Leopoldo, Z., Qi, K., and Morton, D.L. *John Wayne Cancer Institute, Santa Monica, CA 90404.*

We previously reported a significant correlation between clinical evidence of melanoma and the presence of a circulating immune complex (IC) composed of a 90-kD tumor-associated antigen (TA90) and anti-TA90 antibody. In the present study we hypothesized a correlation between TA90-IC and clinical recurrence of melanoma following lymphadenectomy and postoperative adjuvant immunotherapy. We studied 100 melanoma patients who had undergone resection of nodal metastases and postoperative adjuvant therapy with a polyvalent melanoma cell vaccine (PMCV), and from whom serum samples had been obtained after surgical resection but prior to initiation of vaccine therapy. These serum specimens were retrieved from cryopreserved storage, coded, and tested in a blinded manner for TA90-IC. Median follow-up was 25 months (range, 18–78 months). By univariate analysis with log rank test, a positive TA90-IC level was highly correlated with recurrence. Median disease-free survival and 3-year disease-free survival rates were 8 months and 12%, respectively, for the 50 patients with a positive TA90 level, compared with >25 months and >53%, respectively, for the 50 patients with a negative TA90 level ($p=0.0001$). Multivariate analysis with Cox proportional hazard regression considered TA90, number of positive lymph nodes, size of involved lymph nodes, extranodal extension, and disease-free interval: TA90 positivity was the most significant independent variable correlating with disease-free survival ($p=0.0001$). These data indicate that the presence of TA90-IC in patients with no clinical evidence of melanoma postoperatively is highly correlated with subsequent disease recurrence.

# Growth and Characterization of LNCaP Spheroids: A Model used to Optimize Treatment of Micrometastases with Alpha-Particle Emitter, $^{213}$Bi, Labeled Antibodies

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[3]Cornell University Medical Center

# Objectives/Background

Establish a model for optimizing treatment of micrometastases.

- micrometastases are not visualized by conventional imaging
- uptake or targeting can not be assessed
- response is not easily monitored

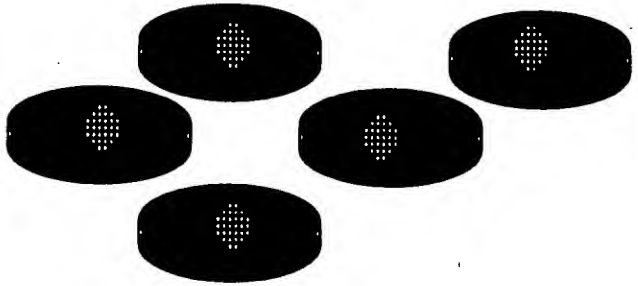
Multicellular spheroids represent an experimental model where individual parameters can be studied separately.

- provide input for model development
- geometry is ideal for determining response to short range alpha particles

- LNCaP cells exhibits properties that are representative of prostate cancer, *in vivo*.

LNCaP multicellular spheroids:

experimental model for study of drug delivery and treatment response parameters to follow are growth delay and PSA production

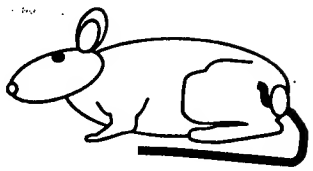


LNCaP cells:

- androgen responsive
  prostatic acid phosphatase (PAP)
  prostate specific antigen (PSA)
  prostate specific membrane antigen (PSMA)
  metastatic potential

Animal tumor models:

difficult to study micrometastatic disease



# Overview

The spheroids have been characterized in terms of:

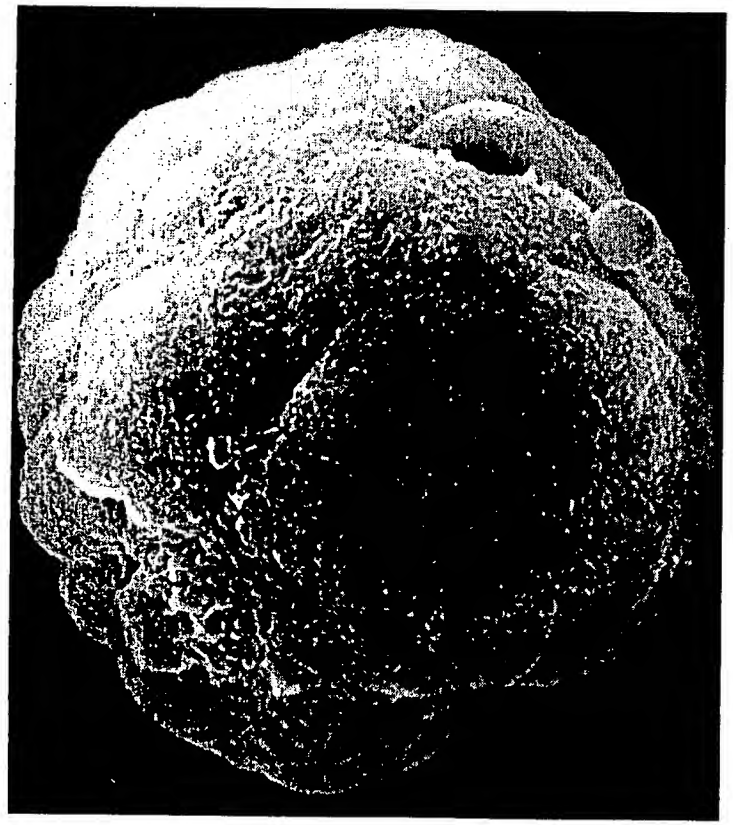
- microscopic structure
- growth kinetic parameters
- proliferating state
- PSA secretion

Preliminary results on response after alpha particle radiation

# Electron Microscope Images

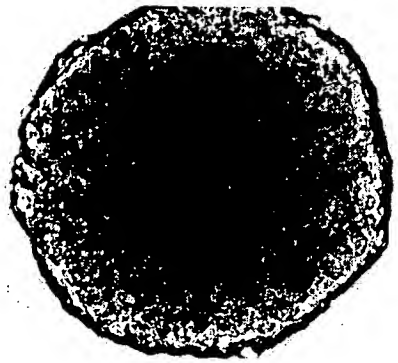
## Scanning EM

45-50 μm-diameter spheroid

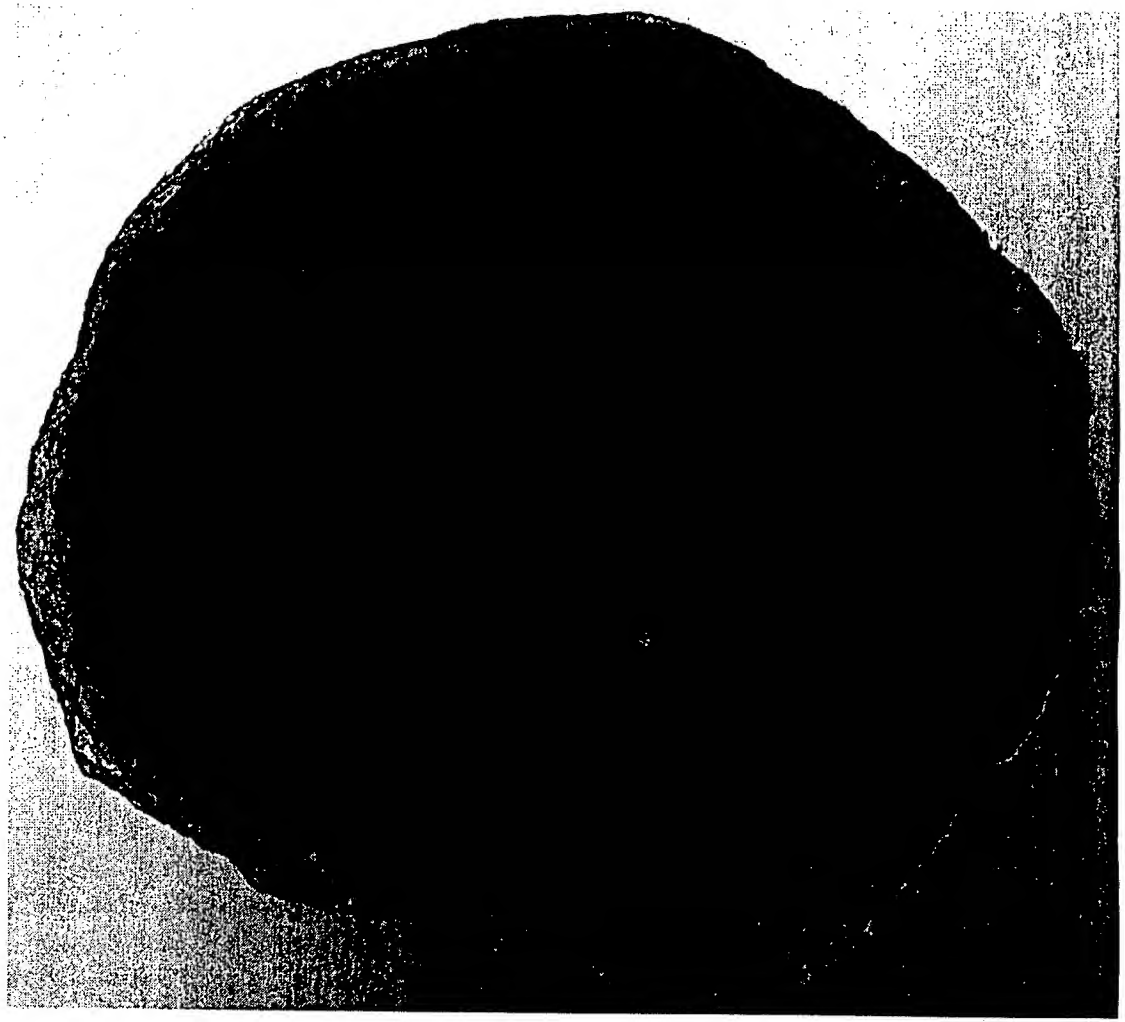


## Transmission EM

# Light Microscope Images

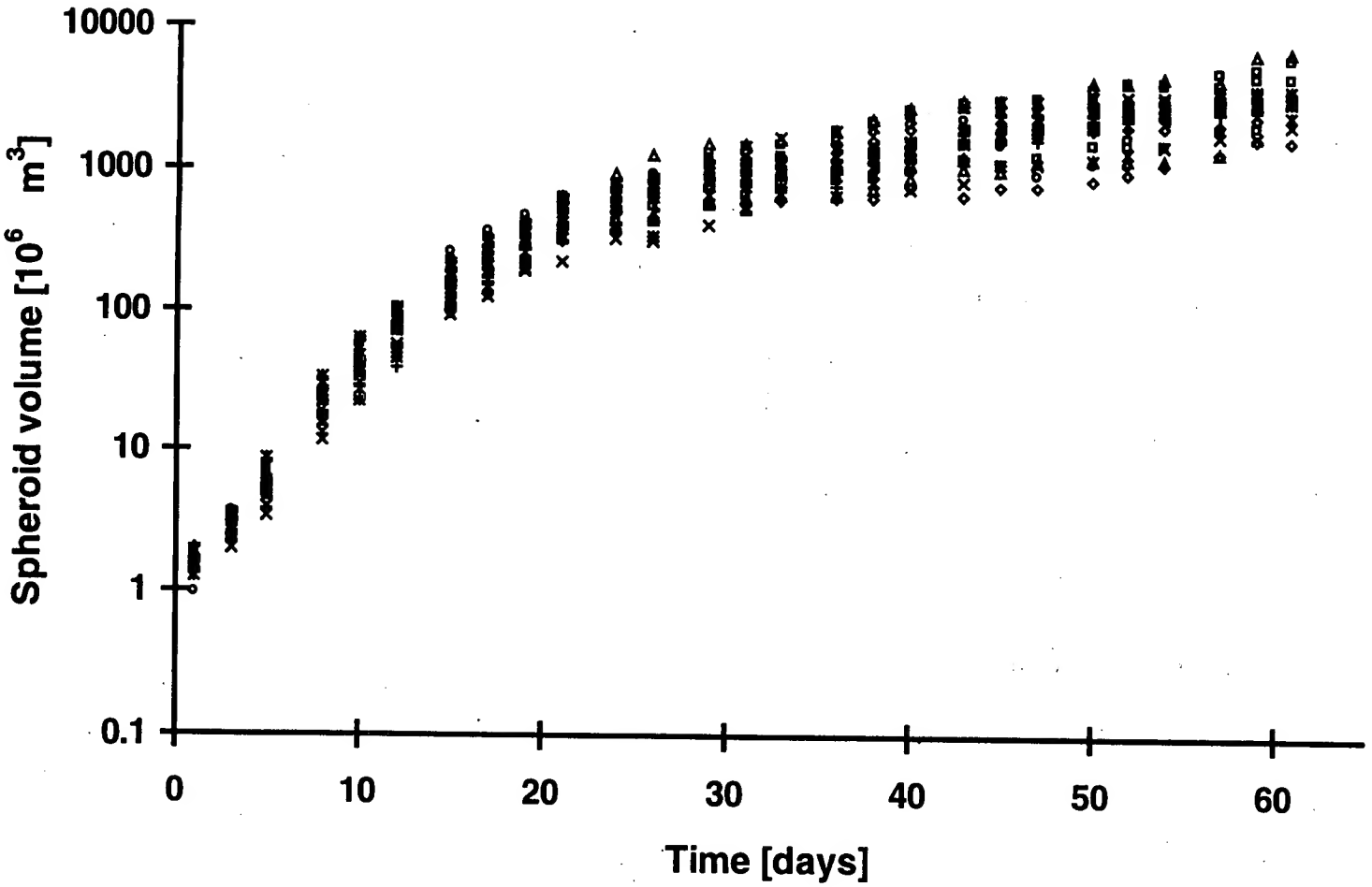


Day 12: Diameter ~ 500 μm



Day 39: Diameter ~ 1500 μm

# LNCaP Spheroid Growth Curves



# LNCaP Spheroid Growth Parameters

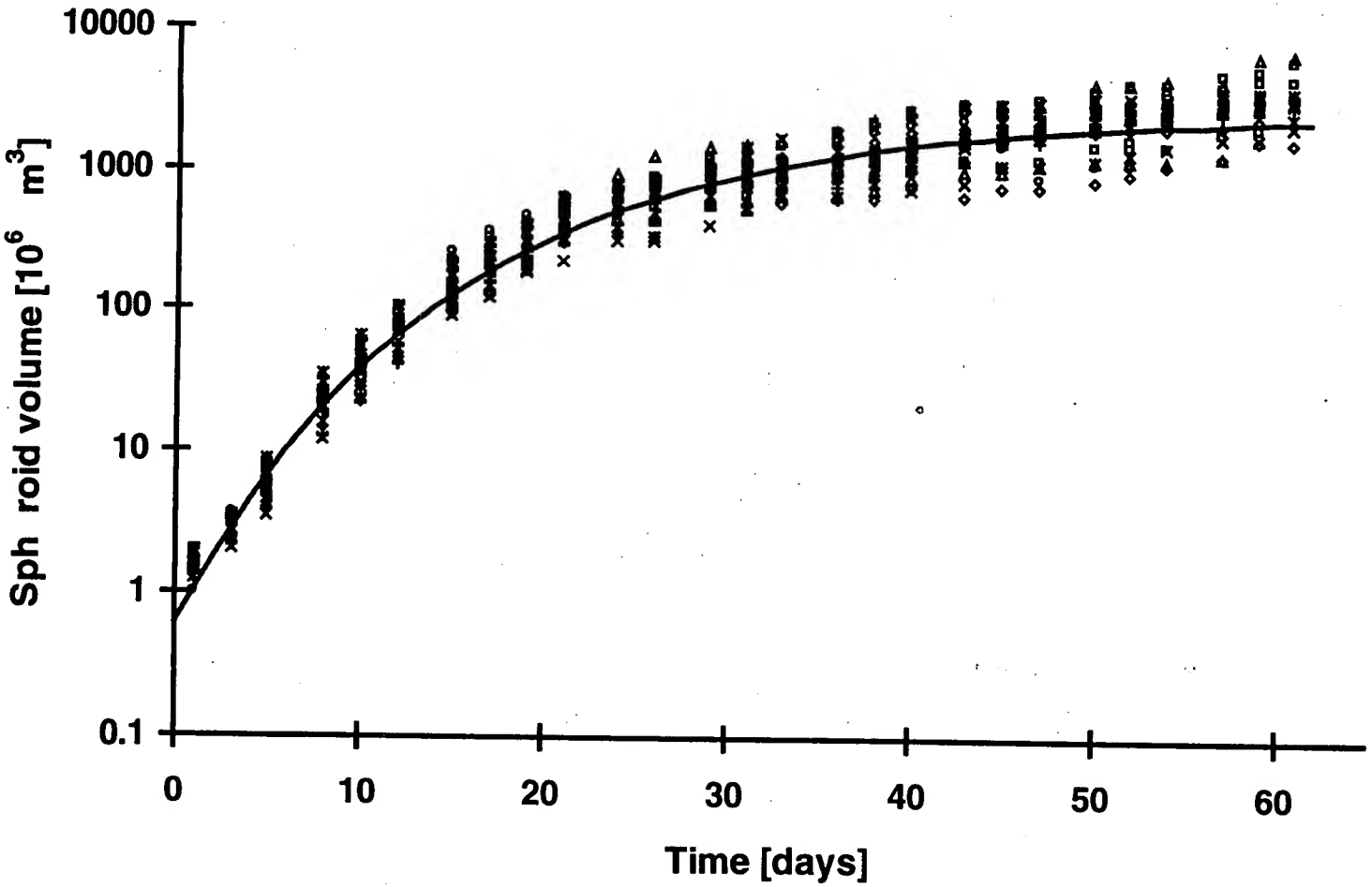
The Gompertzian equation was fitted to spheroid growth curves to determine *a*, *b* and $V_0$.

$$V(t) = V_0 \exp\left(\frac{a}{b}(1-\exp(-bt))\right)$$

$$T = \frac{\ln 2}{a} \qquad V_{\max} = V_0 \exp\left(\frac{a}{b}\right)$$

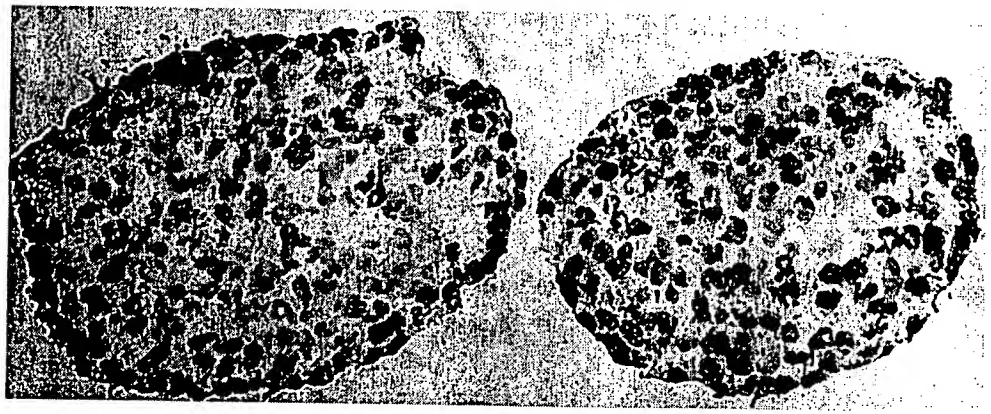
| ***Growth parameters*** | ***LNCaP*** |
|---|---|
| $V_0$ [$10^6$ µm$^3$] | 0.6 ± 0.2 |
| *a* [day$^{-1}$] | 0.57 ± 0.07 |
| *b* [day$^{-1}$] | 0.068 ± 0.008 |
| *T* [h] | 29 ± 4 |
| $V_{max}$ [$10^6$ µm$^3$] | 3078 ± 1277 |

# LNCaP Spheroid Growth Curves

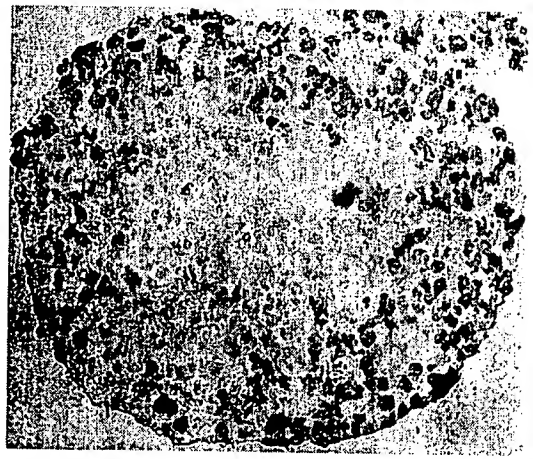


# Light Microscope Images of Spheroid Sections

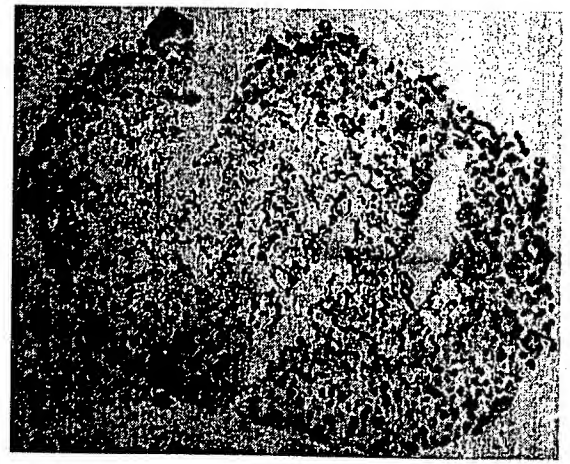
5 μm sections stained for BrdUr (brown) and counter-stained with H & E.
The shell of proliferating cells was determined to 76 ± 13 μm.



200 μm



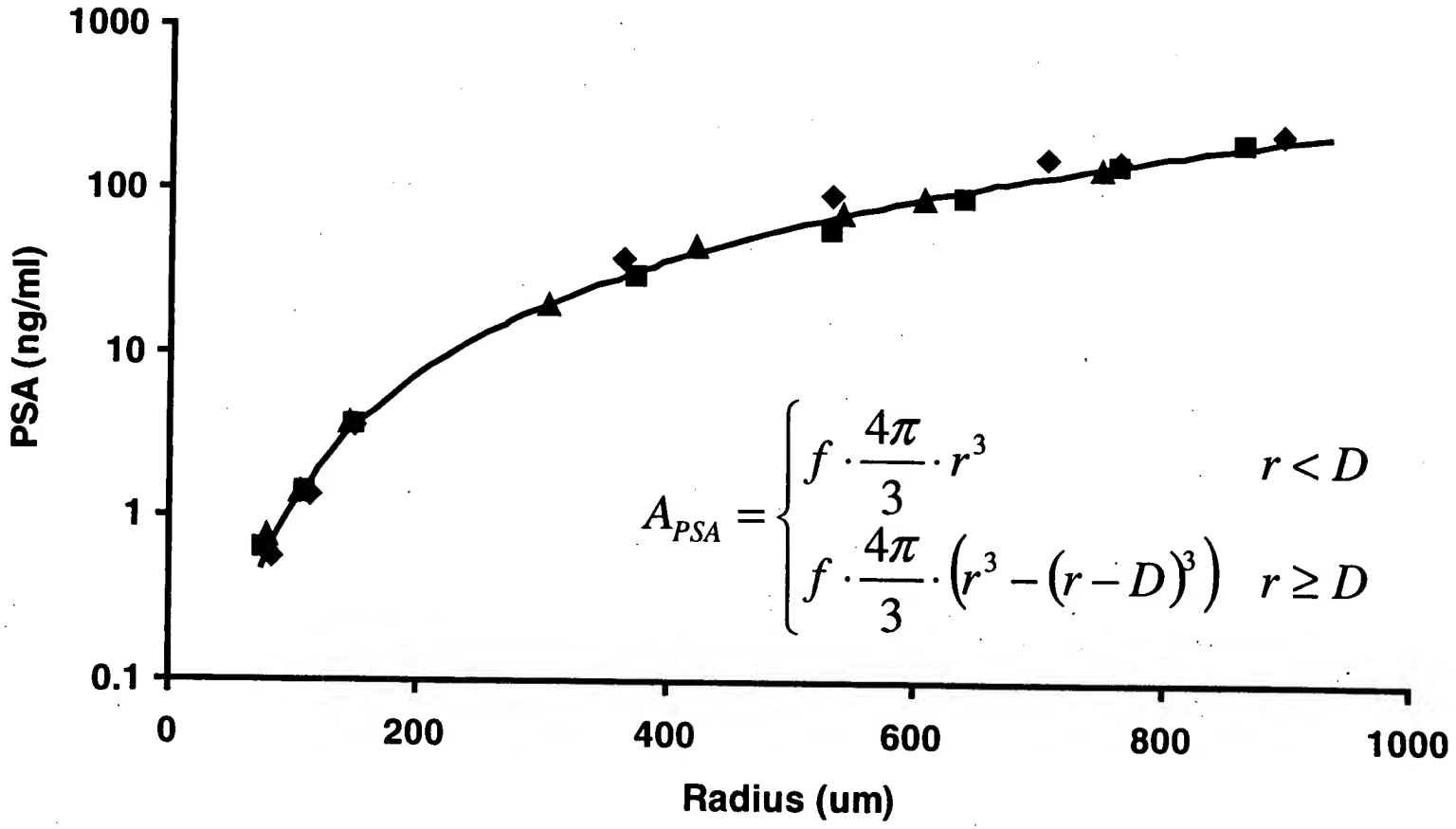
400 μm



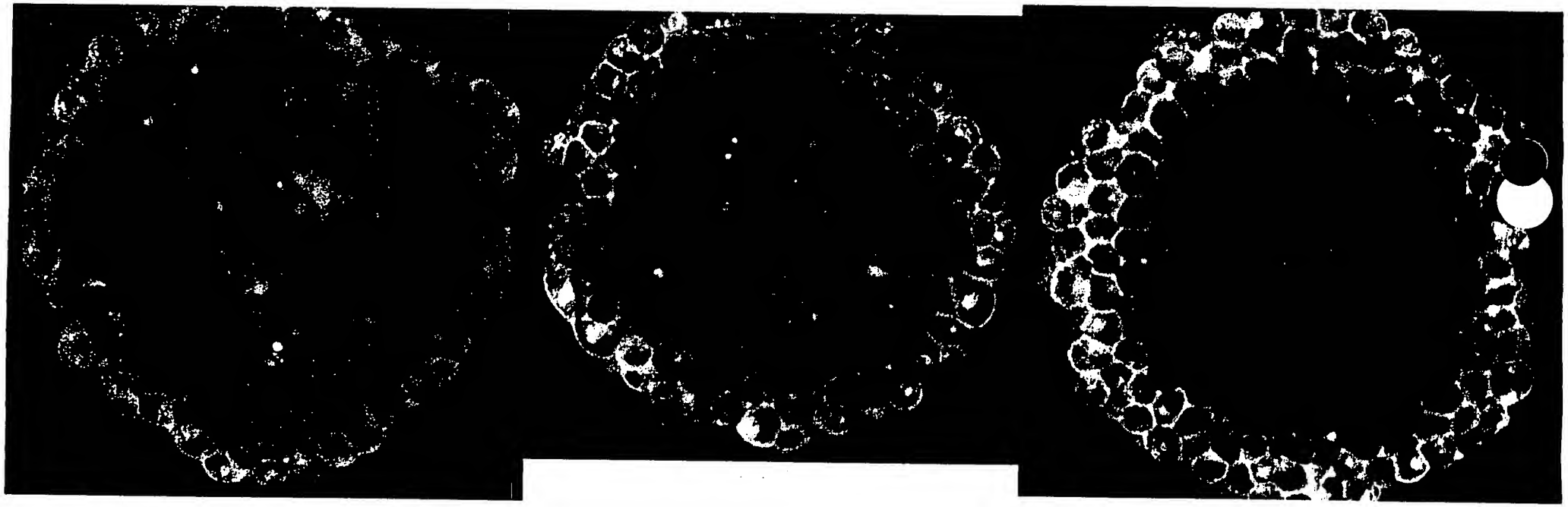
600 μm

# PSA Secretion

The thickness of the shell of PSA producing cells, D, was determined to be 77 ± 3 μm by fitting the equation to the data.



Confocal microscopy slices through the equator of LN3 spheroids following incubation with 10 μg/ml FITC-J591.



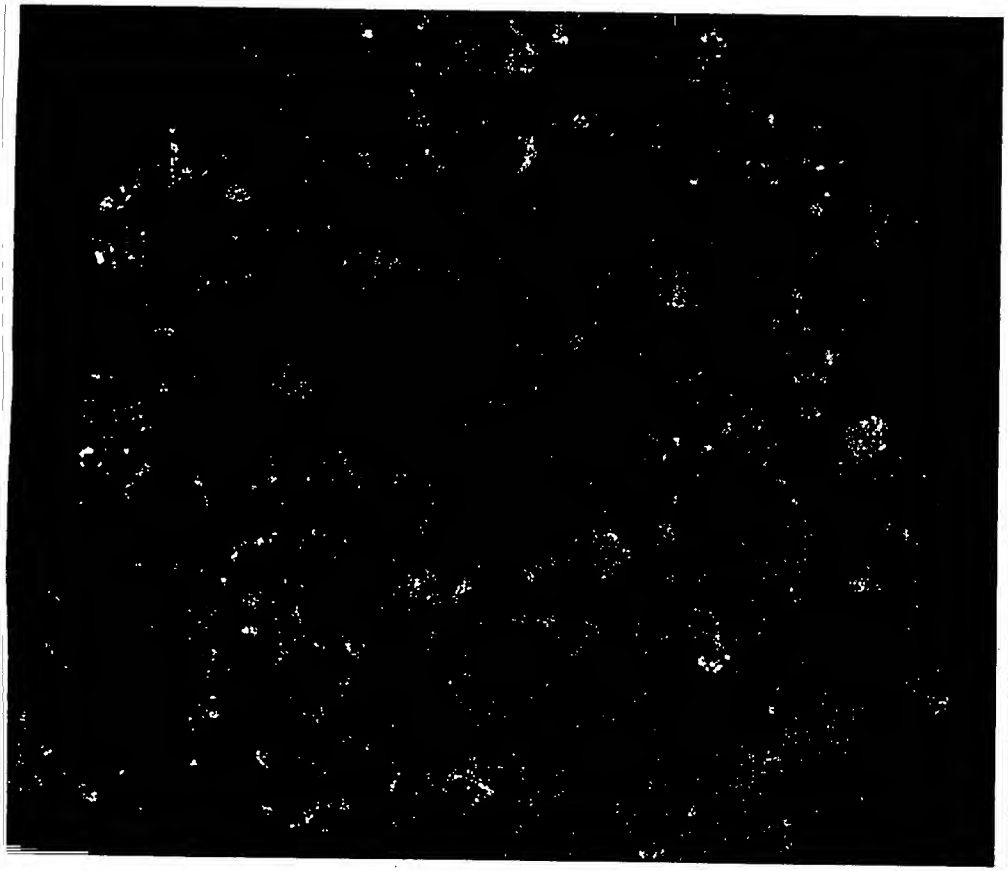
Incubation duration (min):

15 60 120

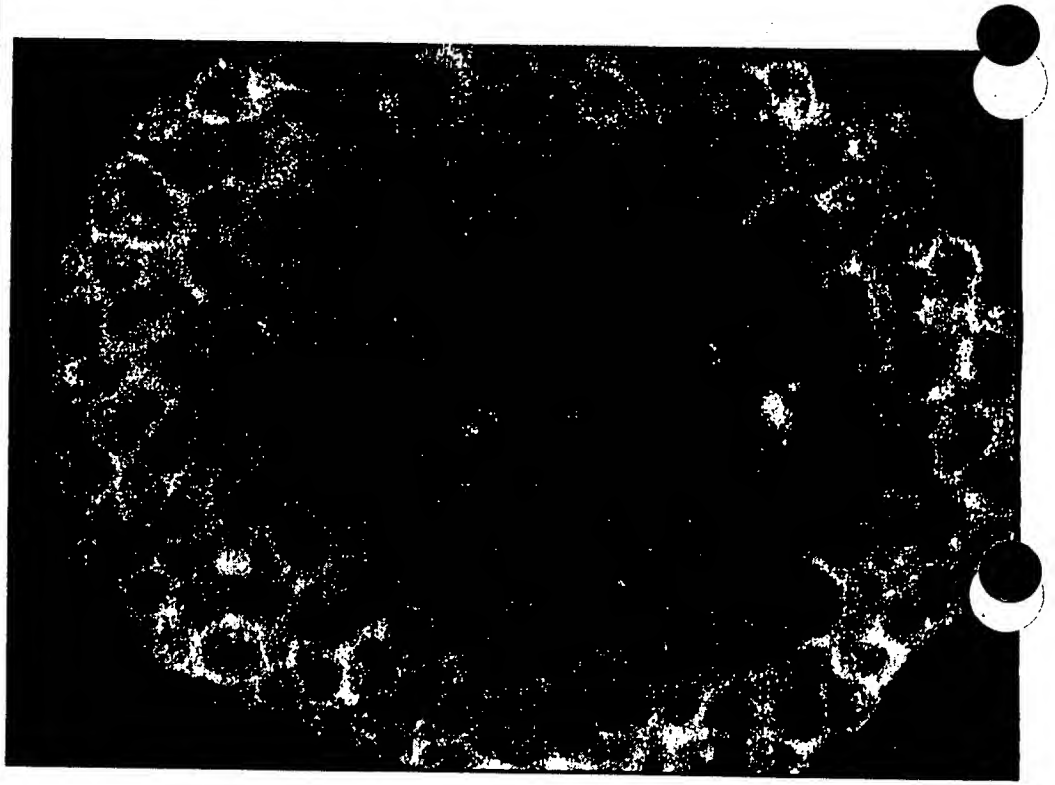
Ab penetration (cell diameters):

1 2 3

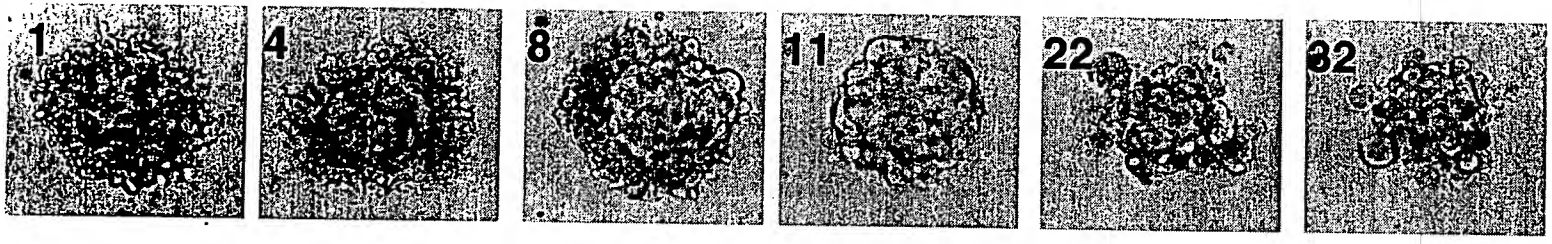
Confocal microscopy slices through the equator of 150 micron-diameter LN3 spheroids after 2 hour incubation with 10 μg/ml FITC-HuM195 (control) or -J591 (specific).



HuM195 (control)

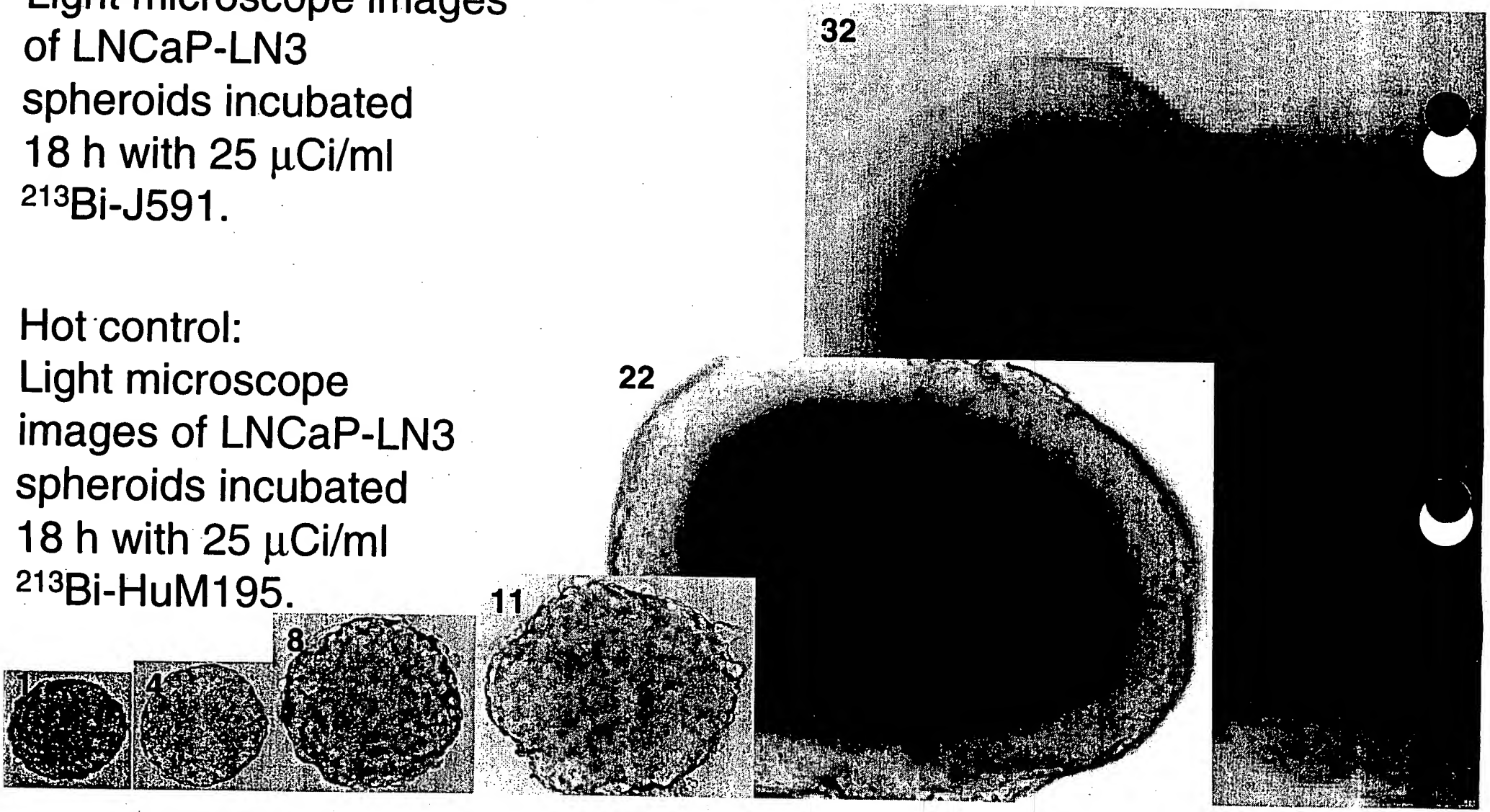


J591 (specific)



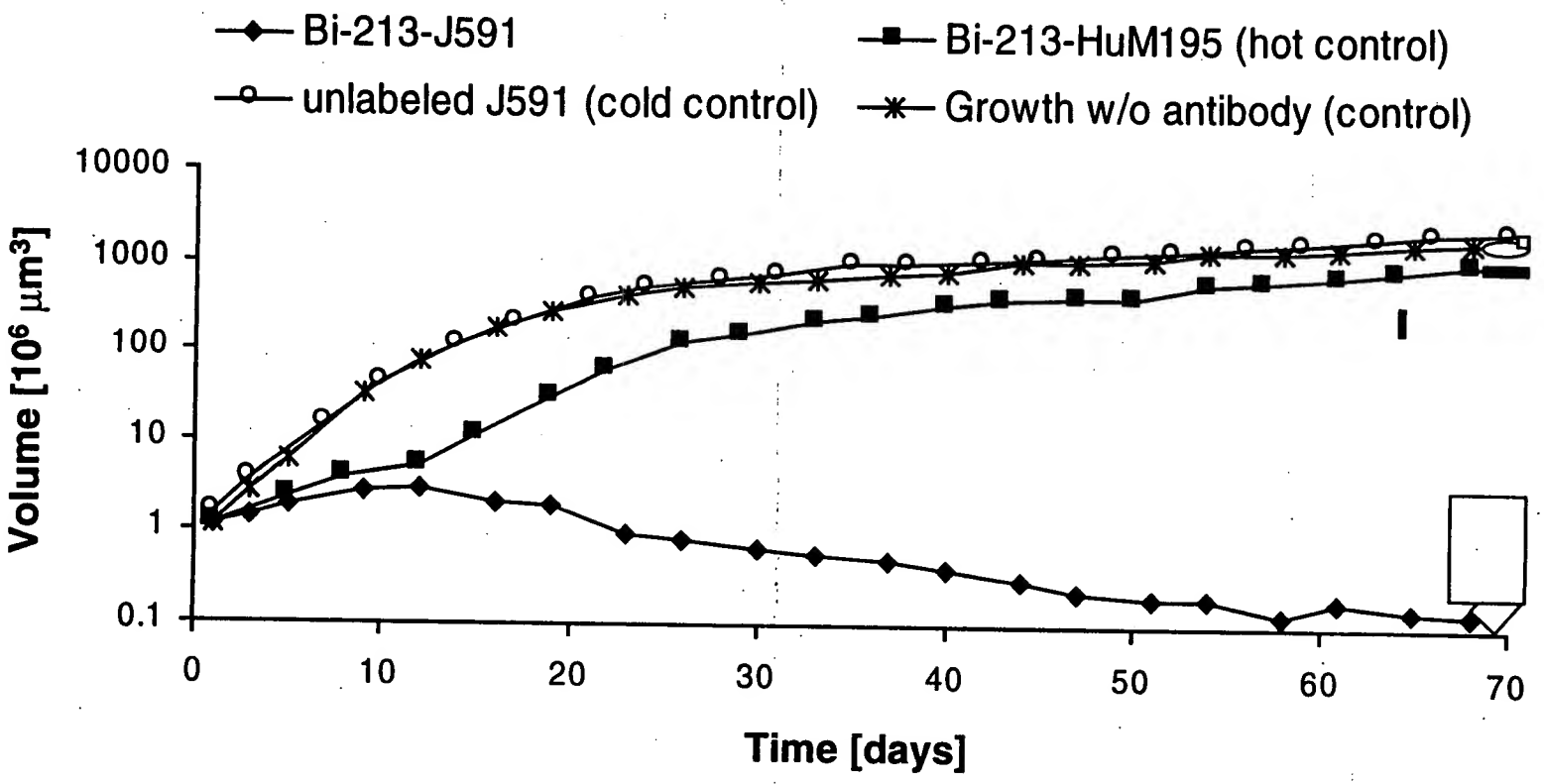

Light microscope images of LNCaP-LN3 spheroids incubated 18 h with 25 μCi/ml $^{213}$Bi-J591.

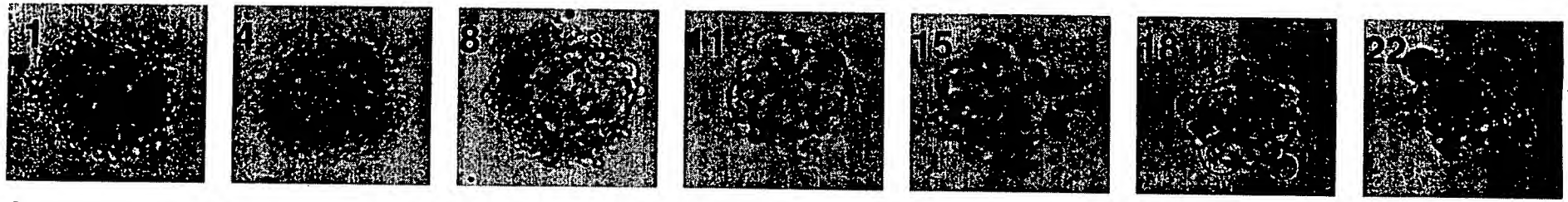
Hot control: Light microscope images of LNCaP-LN3 spheroids incubated 18 h with 25 μCi/ml $^{213}$Bi-HuM195.



# Growth Delay

Median spheroid volume post incubation with 25 μCi/ml $^{213}$Bi.
Diameter on day 0 was approximately 130 μm.





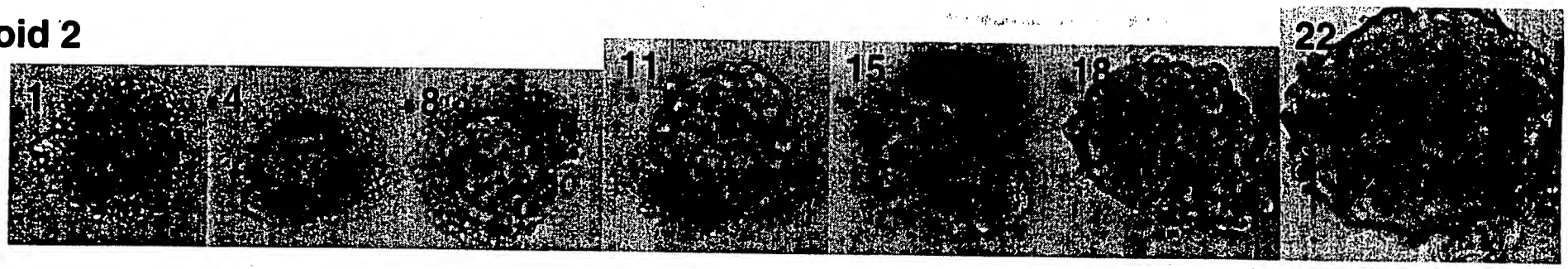

Spheroid 1

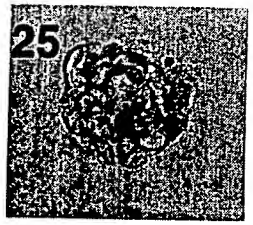
**Light microscope images of LNCaP-LN3 spheroids incubated 18 h with 25 μCi/ml $^{213}$Bi-J591.**

**The top and bottom panels show examples of spheroid break-up (top) and growth delay (bottom).**

**A prolonged growth delay relative to hot controls was seen in all treated spheroids.**

Spheroid 2

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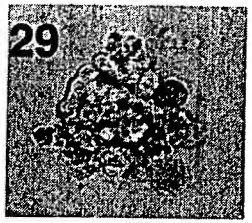
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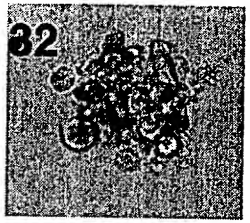
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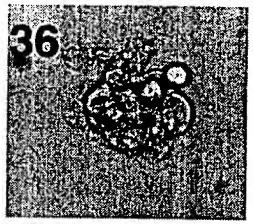
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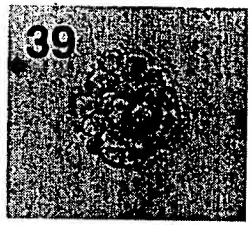
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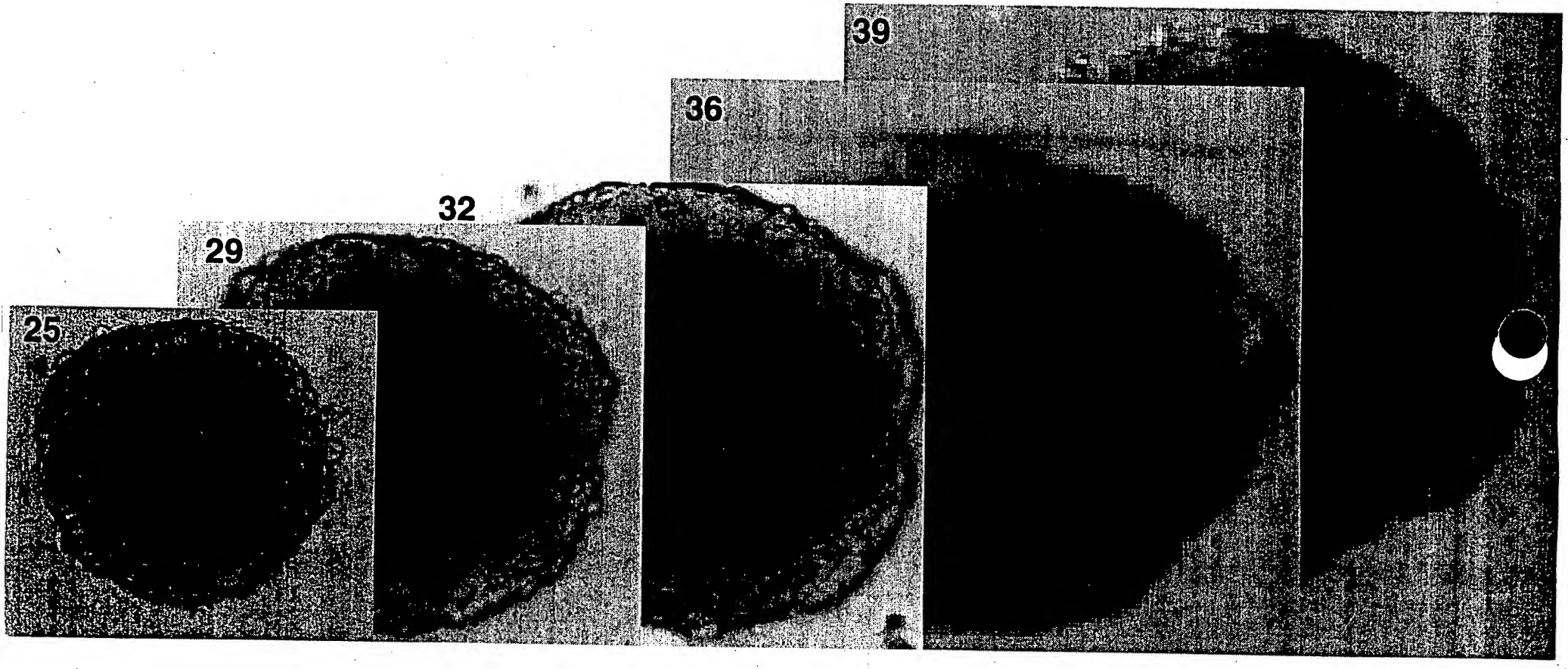

39
36
32
29
25

# The LNCaP spheroid model...

represents a well characterized model for prostate cancer.

- is well suited for studies of the efficacy of alpha-particles since alphas have short range.

  is relevant for antibody penetration studies and to investigate targeting of micrometastatic disease.

  is an experimental analog which will help develop mathematical models to assist the development of optimal clinical therapy. This will be important for treatment of micrometastases where targeting and response cannot be easily monitored in patients.

(247 µCi, 27.3 µg Ab, 2ml)

Graph 5. $^{90}$Y-HuM195, 5 h, without Dexamethasone

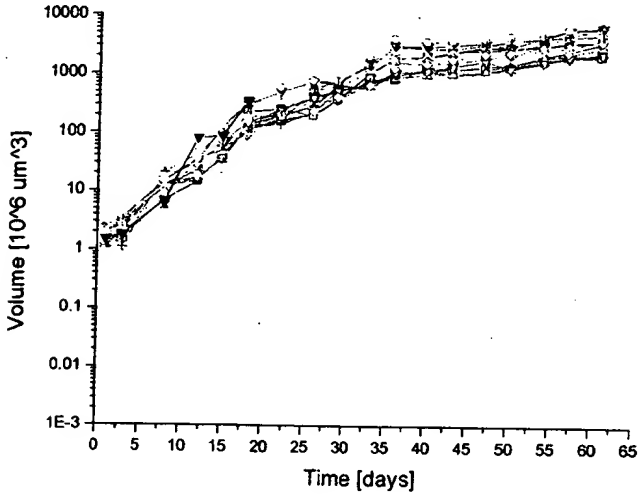
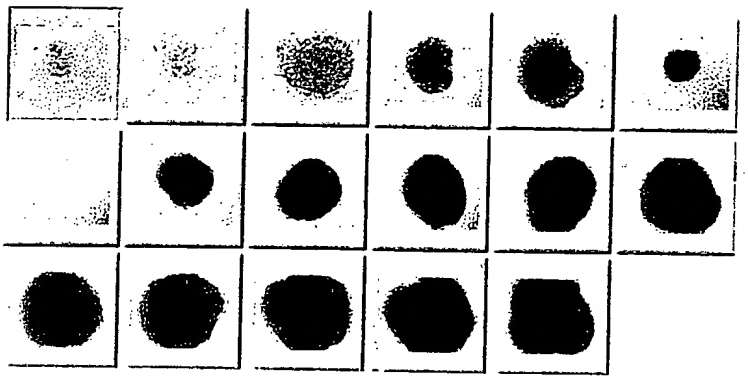



Figure 5. $^{90}$Y-HuM195, 5 h, without Dexamethasone*



Graph 6. $^{90}$Y-HuM195, 5 h, with Dexamethasone

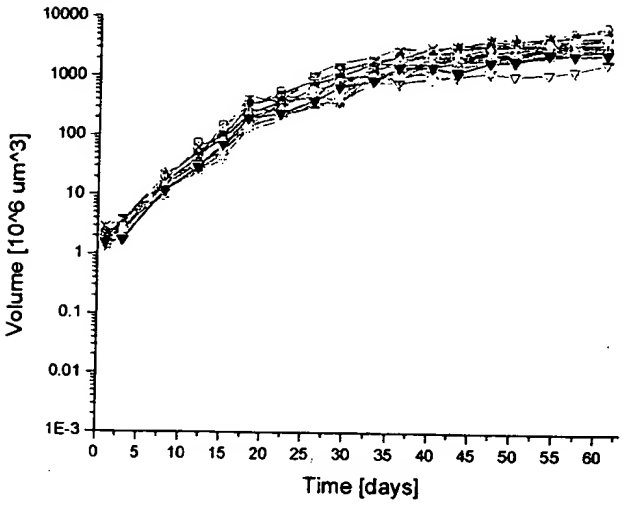
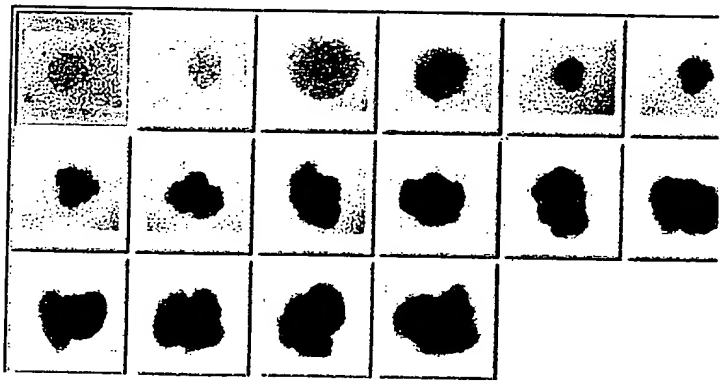
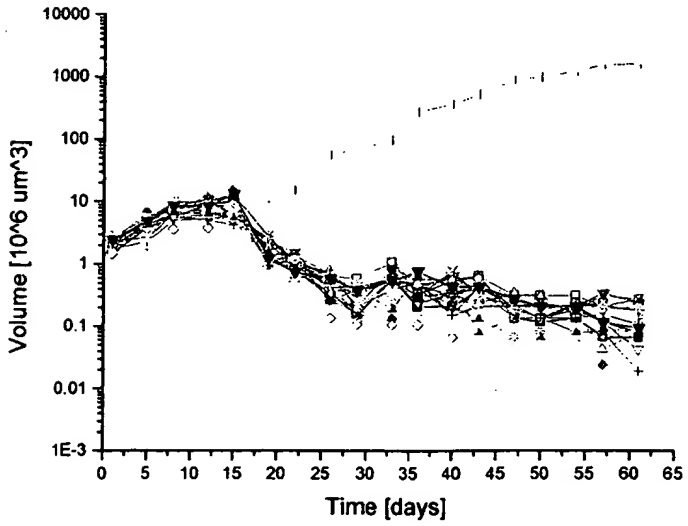



Figure 6. $^{90}$Y-HuM195, 5 h, with Dexamethasone*_



(247 µCi, 27.3 µg Ab, 2ml)

Graph 7. $^{90}$Y-HuM195, 19 h, without Dexamethasone




Graph 8. $^{90}$Y-HuM195, 19 h, with Dexamethasone

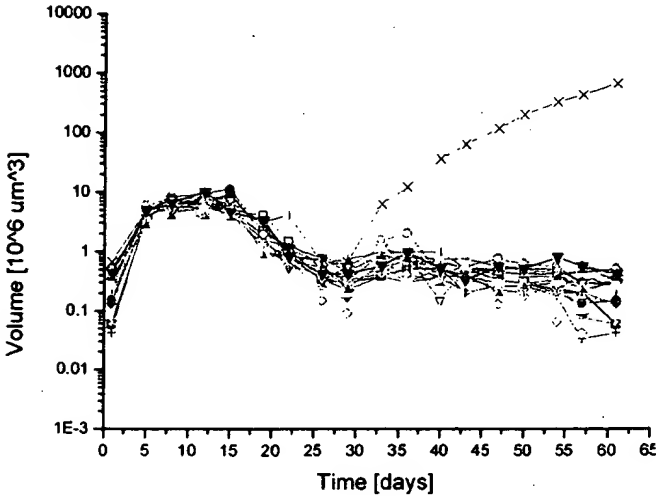



Figure 7. $^{90}$Y-HuM195, 19 h, without Dexamethasone*

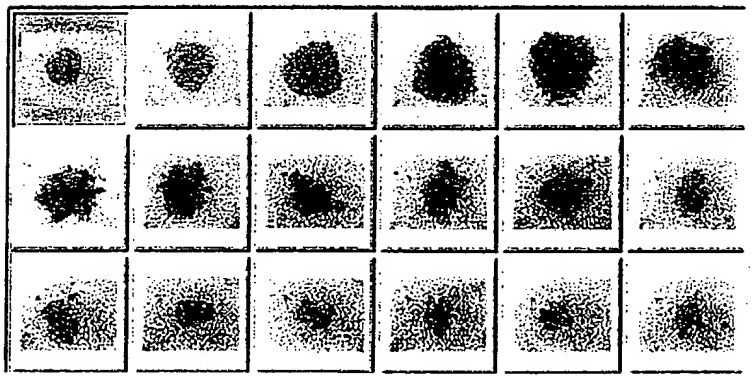
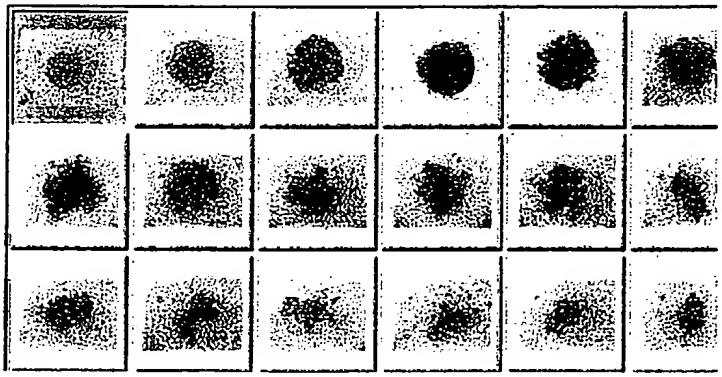
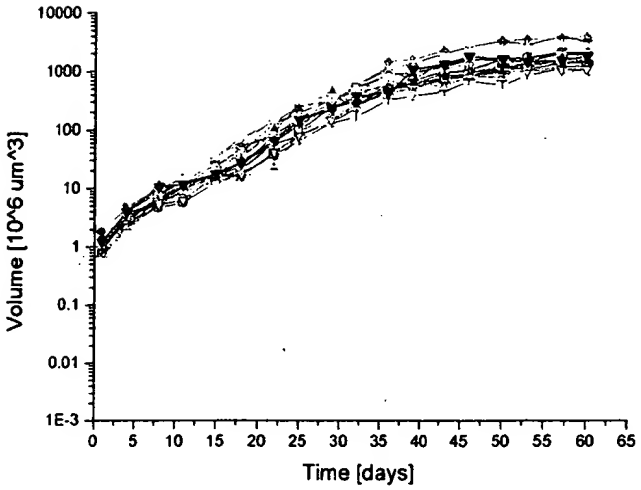


Figure 8. $^{90}$Y-HuM195, 19 h, with Dexamethasone*



Graph 9. External Beam, 6 Gy, without Dexamethasone




Graph 10. External Beam, 6 Gy, with Dexamethasone

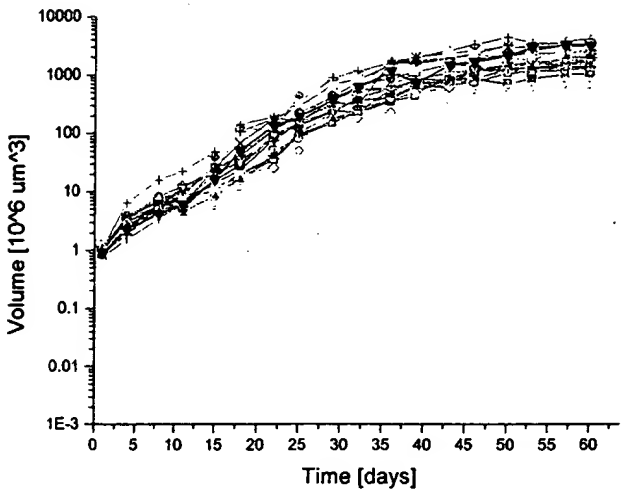



Figure 9. External Beam, 6 Gy, without Dexamethasone*

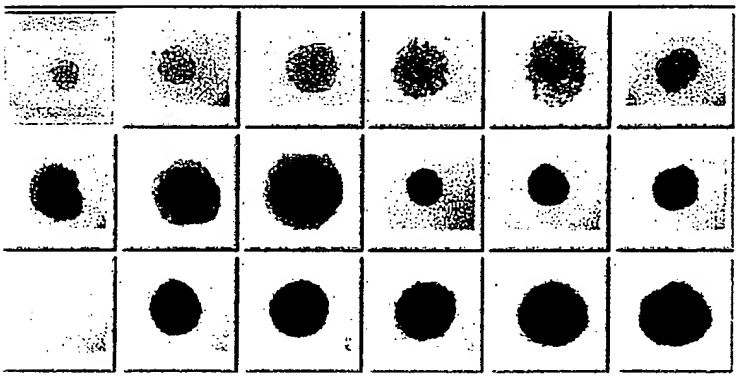
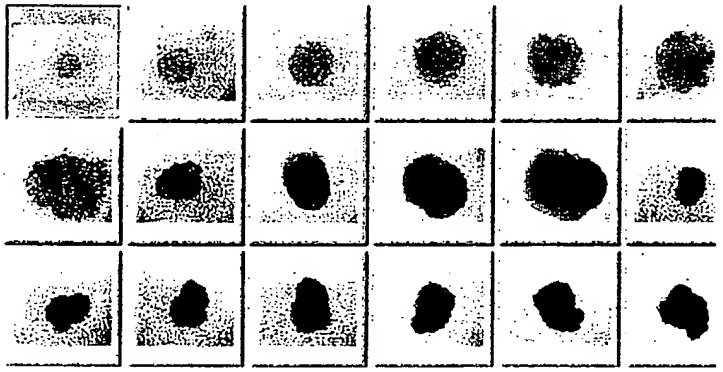
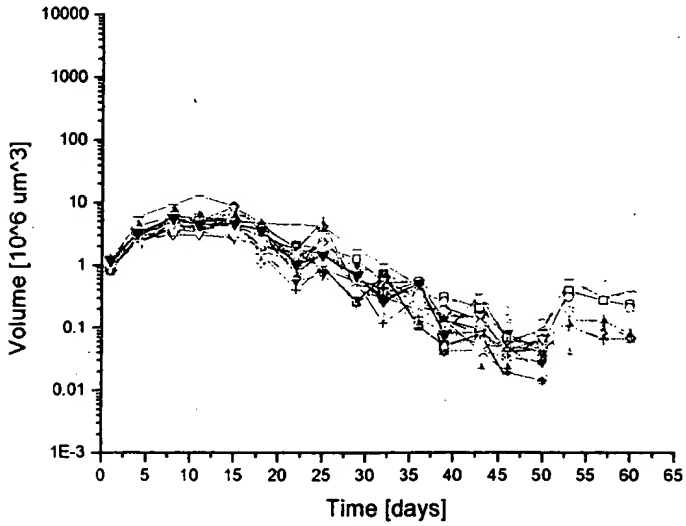


Figure 10. External Beam, 6 Gy, with Dexamethason



Graph 11. External Beam, 12 Gy, without Dexamethasone




Graph 12. External Beam, 12 Gy, with Dexamethasoı

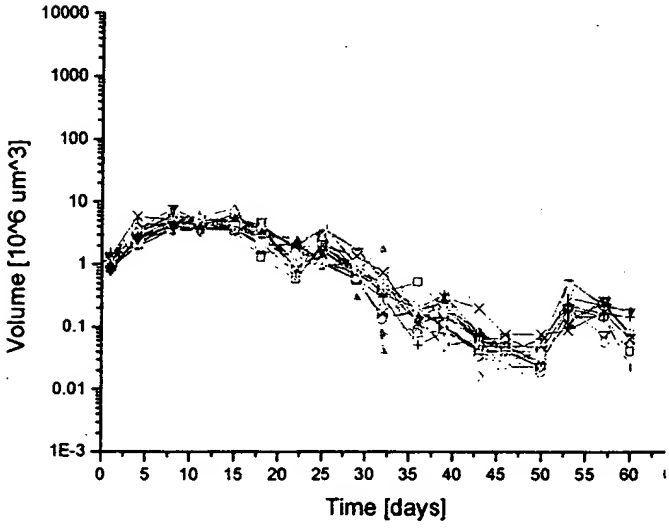



Figure 11. External Beam, 12 Gy, without Dexamethasone*

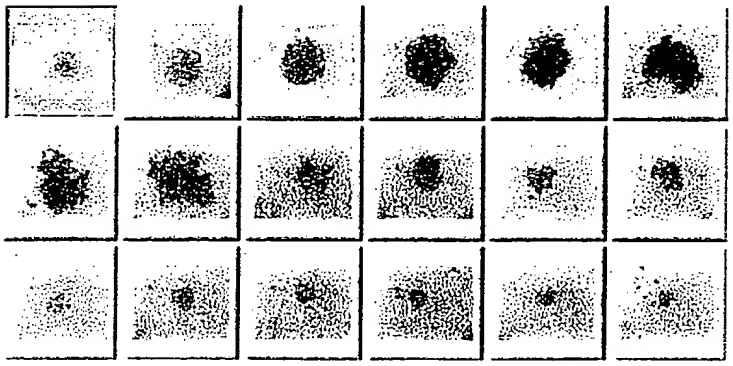
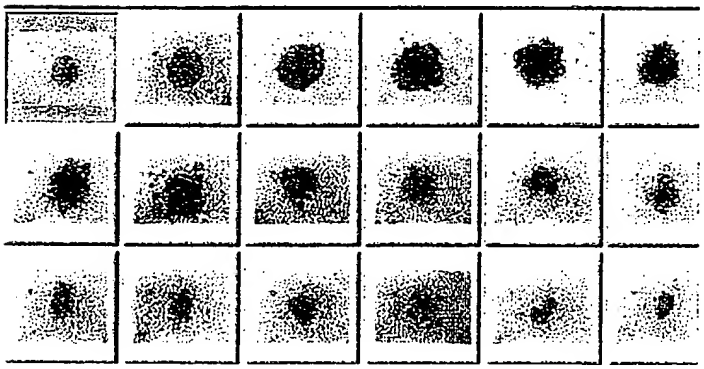


Figure 12. External Beam, 12 Gy, with Dexamethasoı



(247 µCi, 27.3 µg Ab, 2ml)

Graph 13. $^{90}$Y-J591, 5 h, without Dexamethasone

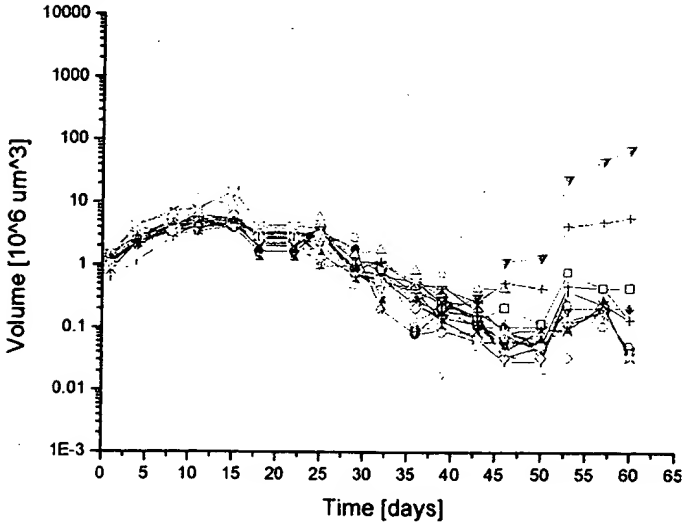
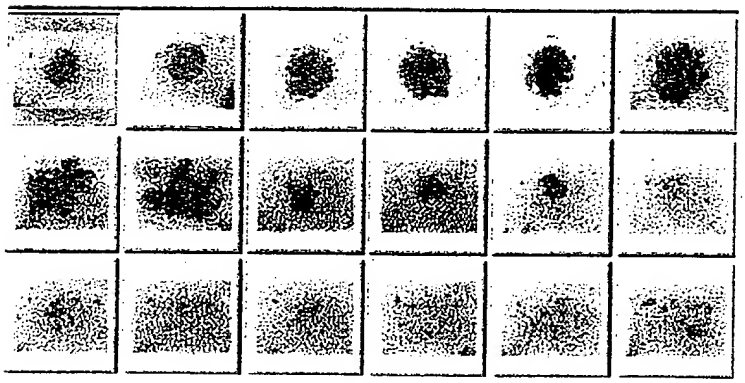



Figure 13. $^{90}$Y-J591, 5 h, without Dexamethasone*



Graph 14. 90Y-J591, 5 h, with Dexamethasone

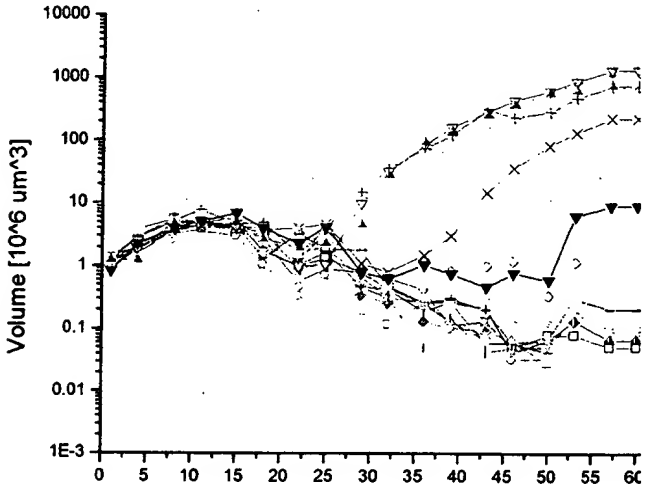
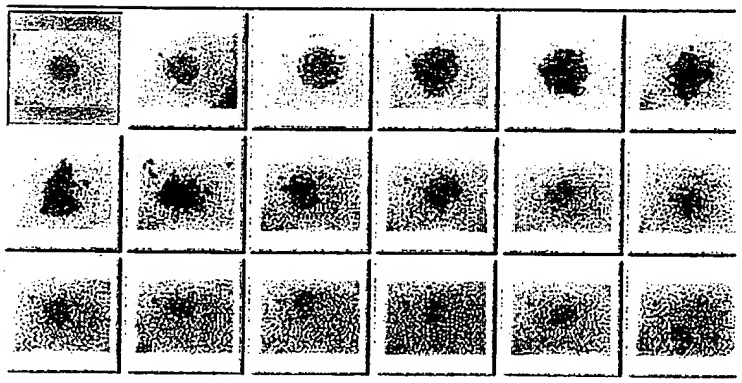



Figure 14. 90Y-J591, 5 h, with Dexamethasone (Days 1 to 32)



(247 μCi, 27.3 μg Ab, 2ml)

Graph 15. $^{90}$Y-J591, 19 h, without Dexamethasone

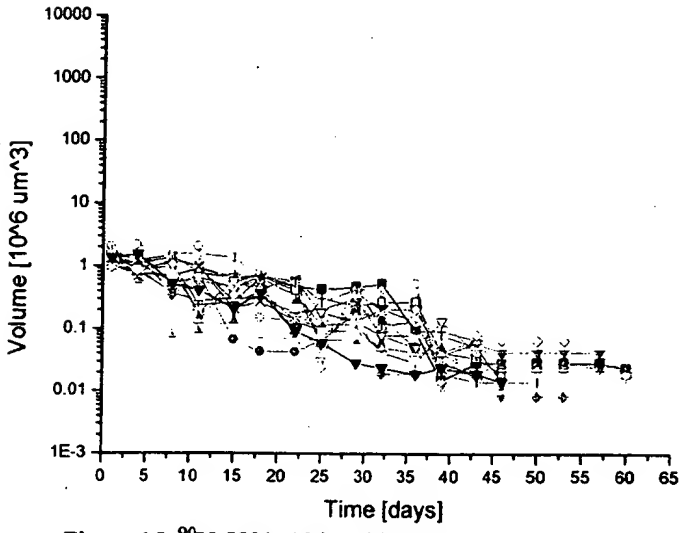



Figure 15. $^{90}$Y-J591, 19 h, without Dexamethasone*

Graph 16. 90Y-J591, 19 h, with Dexamethasone

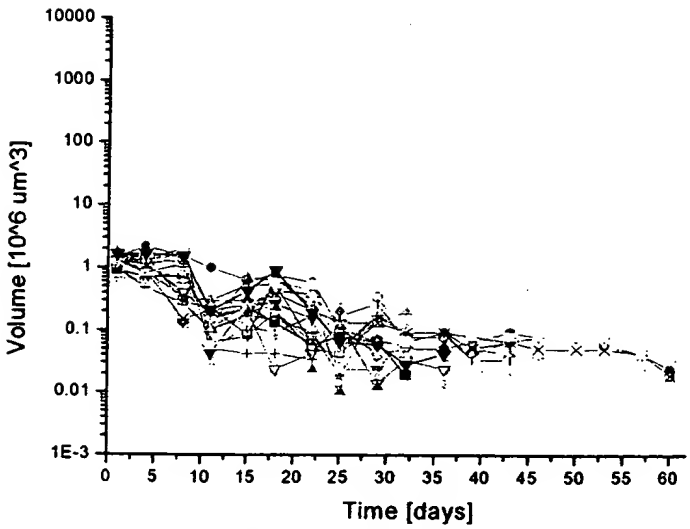
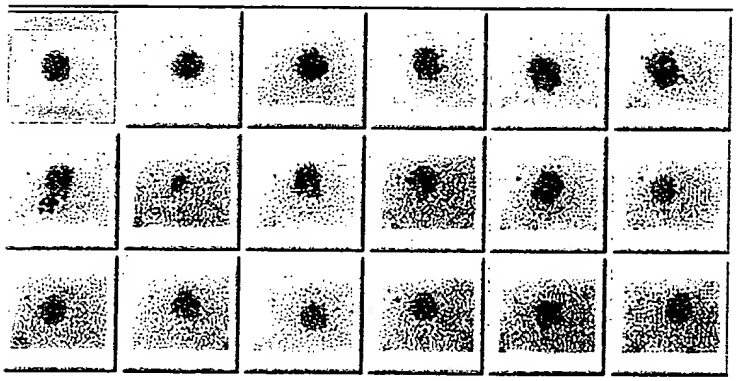
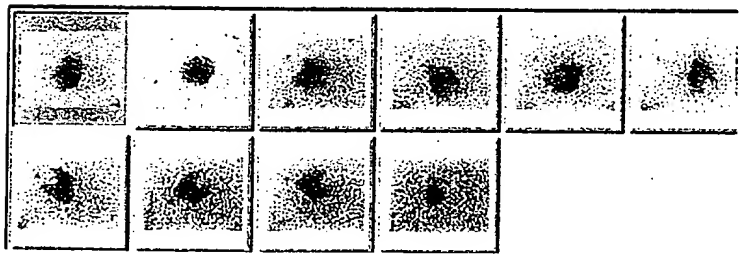
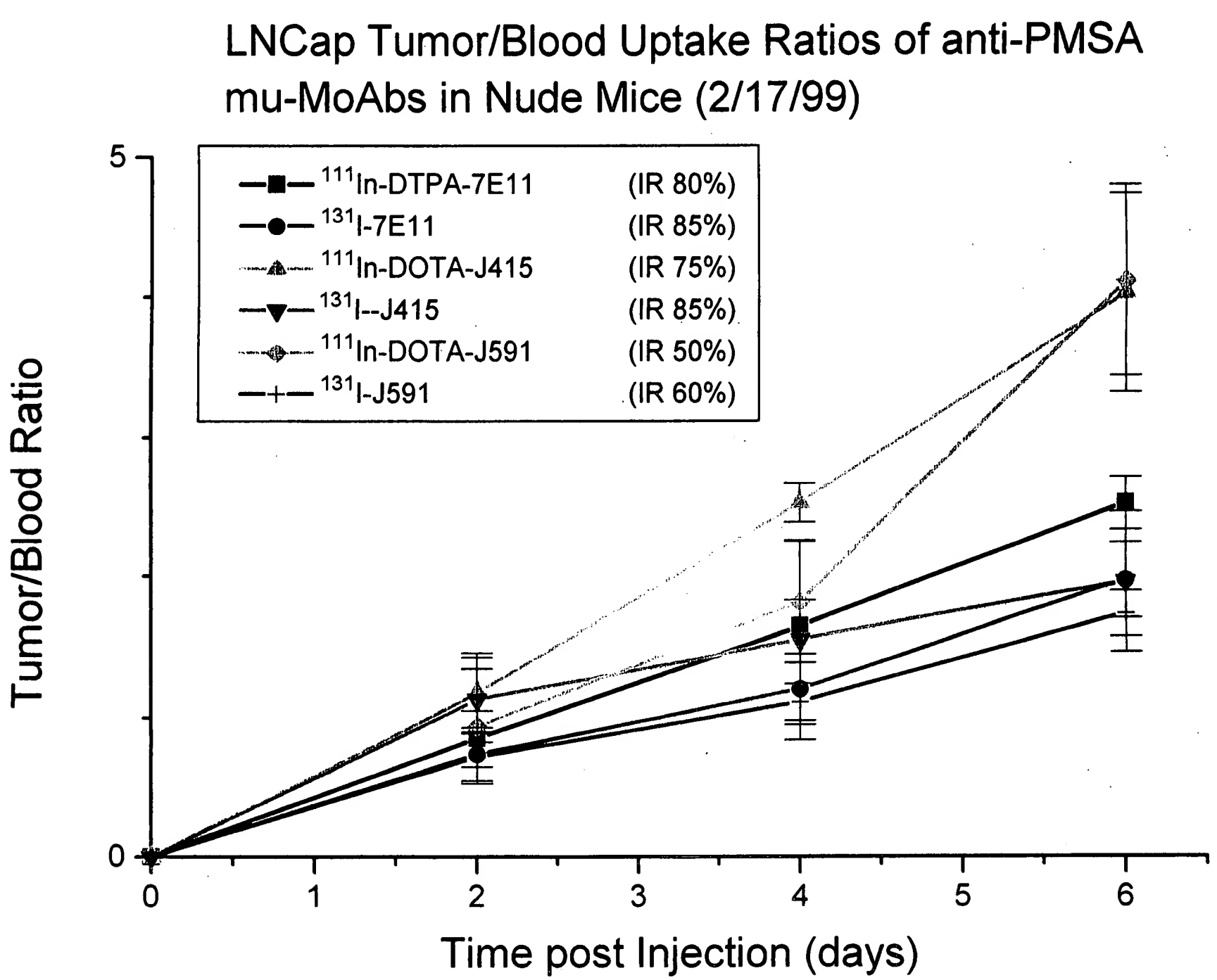


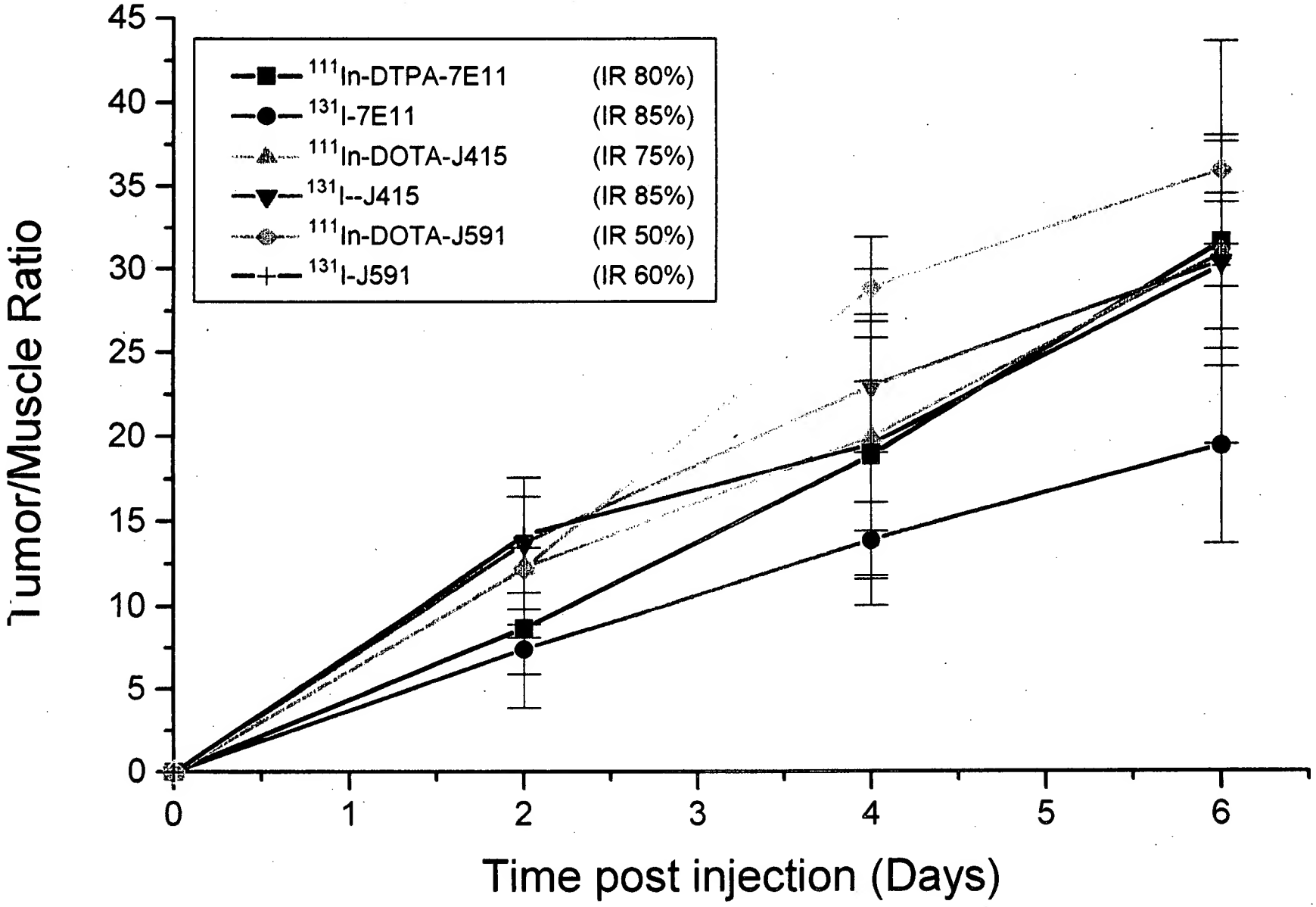

Figure 16. 90Y-J591, 19 h with Dexamethasone (Days 1 to 32)

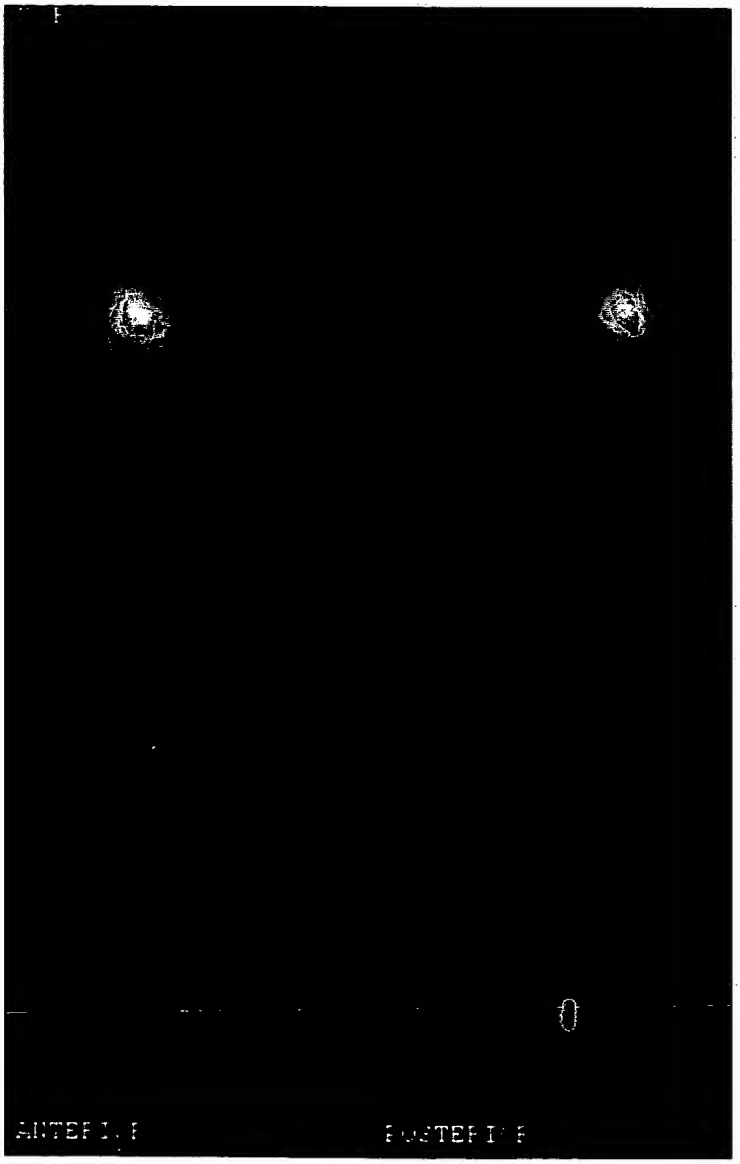


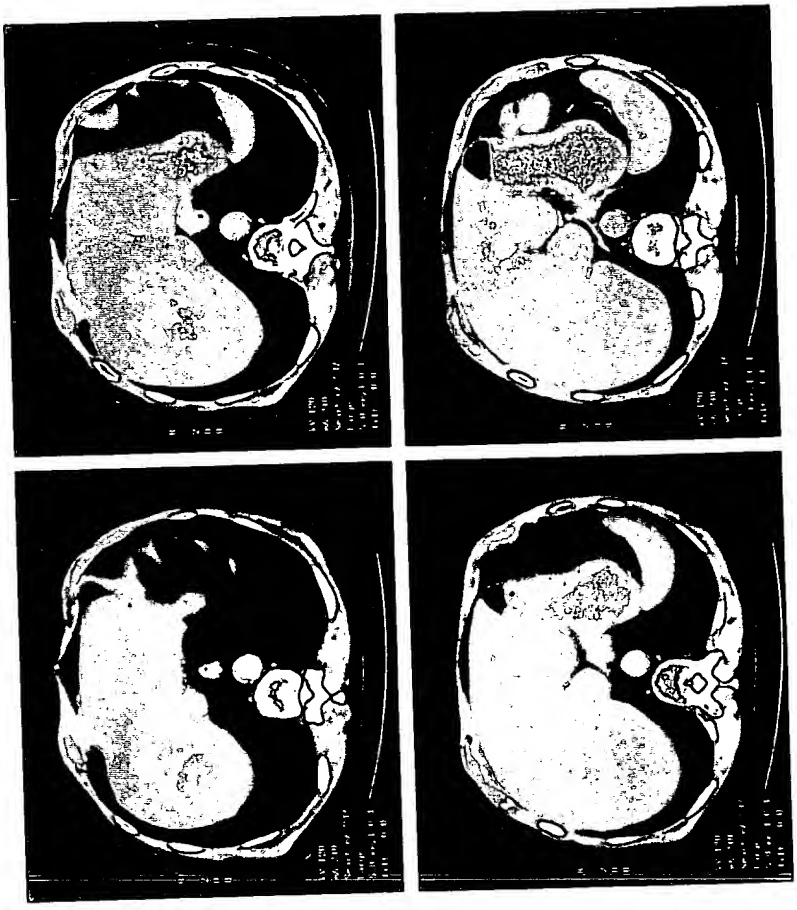



LNCap Tumor/Blood Uptake Ratios of anti-PMSA mu-MoAbs in Nude Mice (2/17/99)
111In-DTPA-7E11 (IR 80%)
131I-7E11 (IR 85%)
111In-DOTA-J415 (IR 75%)
131I--J415 (IR 85%)
111In-DOTA-J591 (IR 50%)
131I-J591 (IR 60%)
Tumor/Blood Ratio
0
5
0
1
2
3
4
5
6
Time post Injection (days)

# Tumor/Muscle Ratios of anti-PMSA mu-MoAbs in Nude Mice bearing LNCap Tumors (2/17/99)







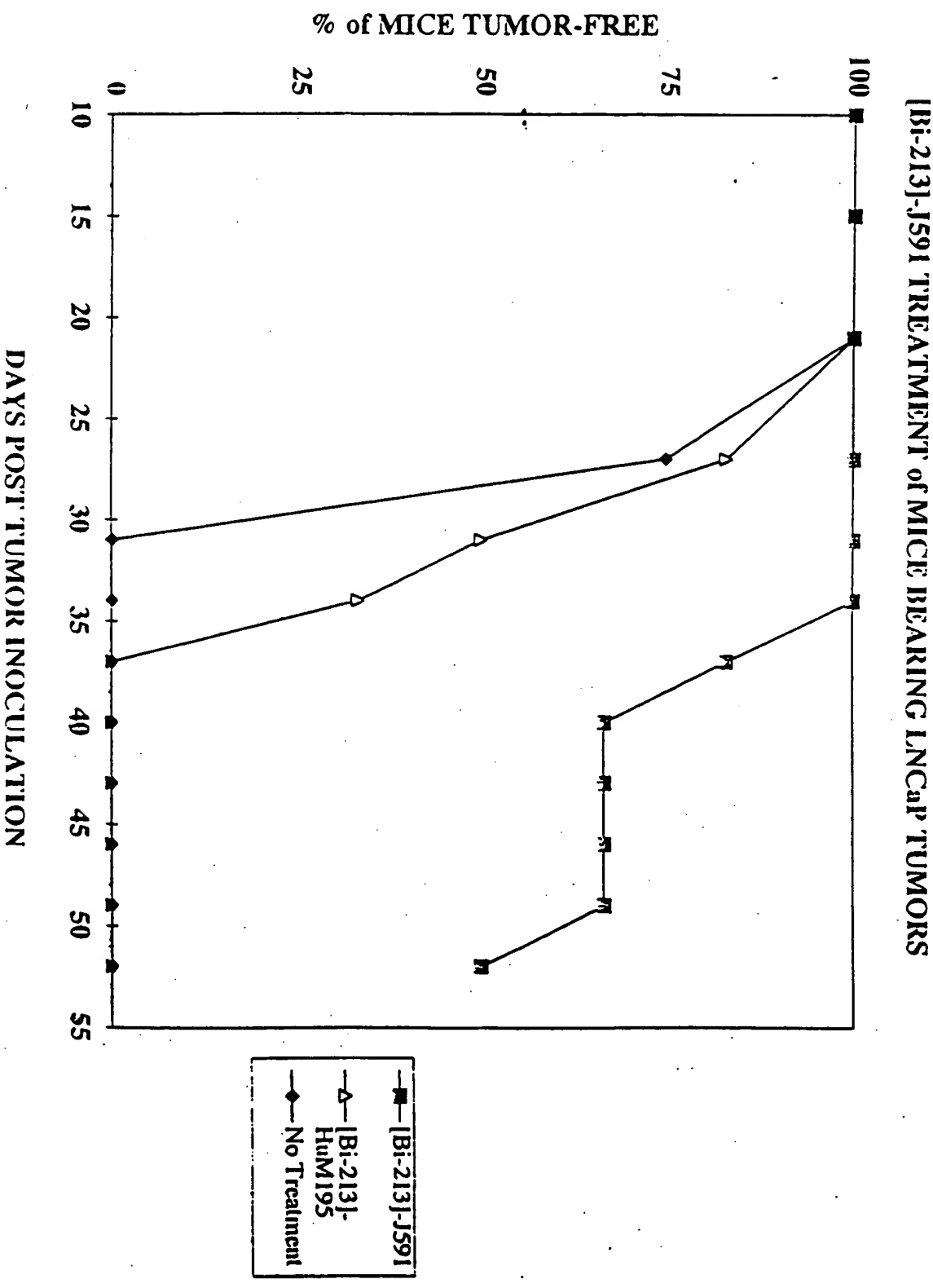

[Bi-213]-J591 TREATMENT of MICE BEARING LNCaP TUMORS
% of MICE TUMOR-FREE
0
25
50
75
100
10
15
20
25
30
35
40
45
50
55
DAYS POST TUMOR INOCULATION
[Bi-213]-J591
[Bi-213]-HuM195
No Treatment

Fig. 1

# PSA VALUES in MICE BEARING LNCaP TUMORS

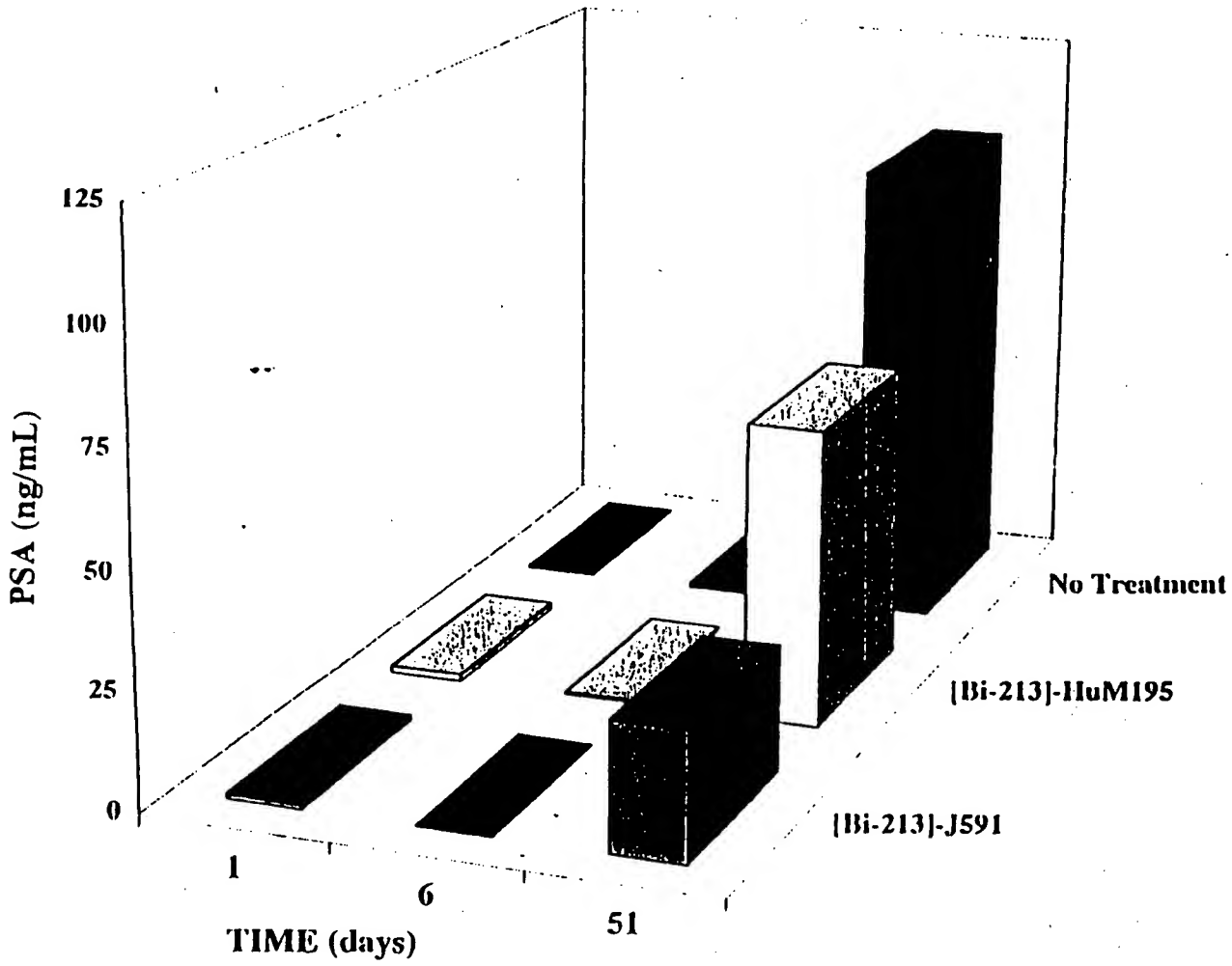



Fig. 2

(2)

# ADCC (LNCAP's) with Antibody [4 ug/ml]

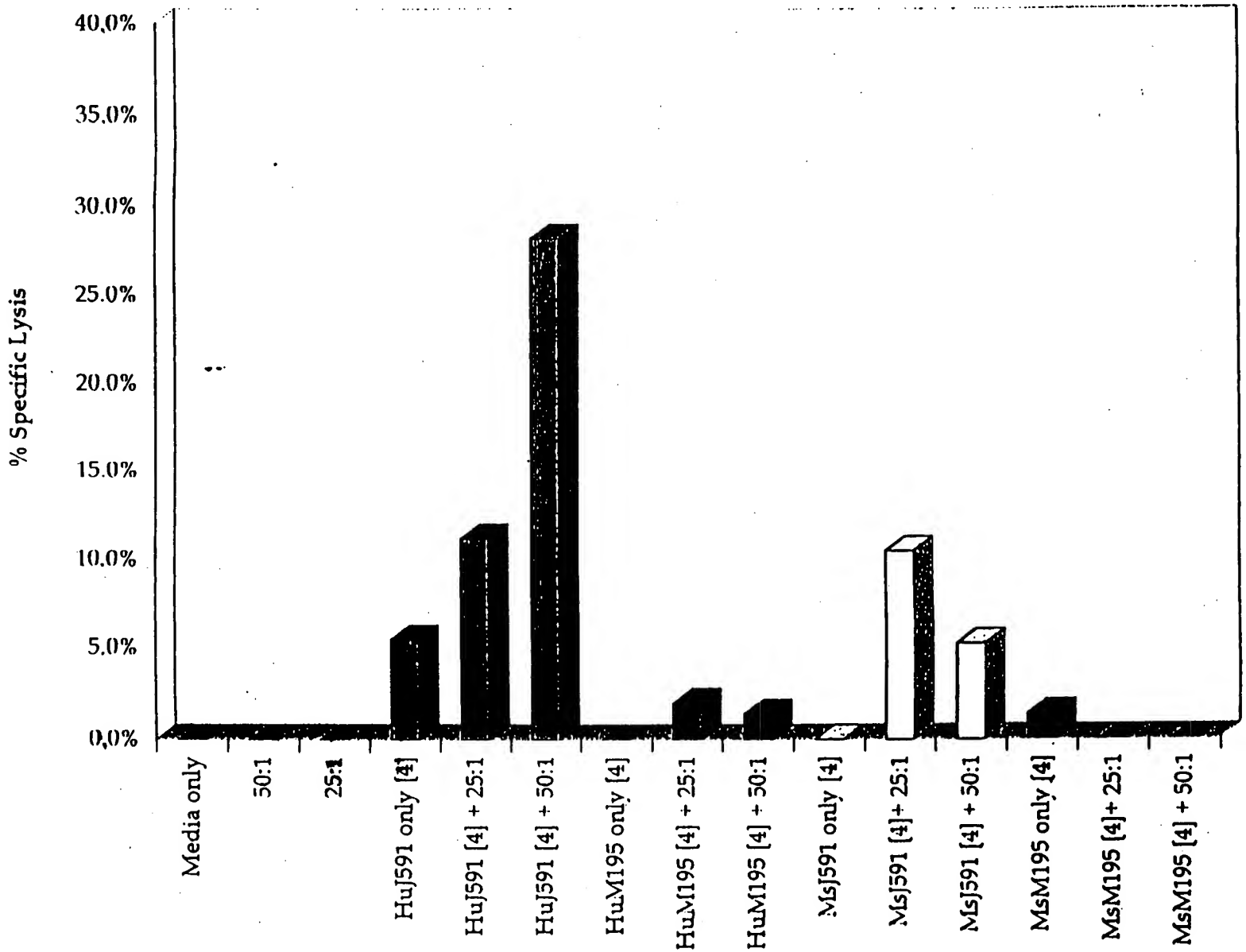



Fig. 3

# ADCC (LNCAP's) with Various Antibodies at Different Concentrations & E:T of 50:1 & 25:1

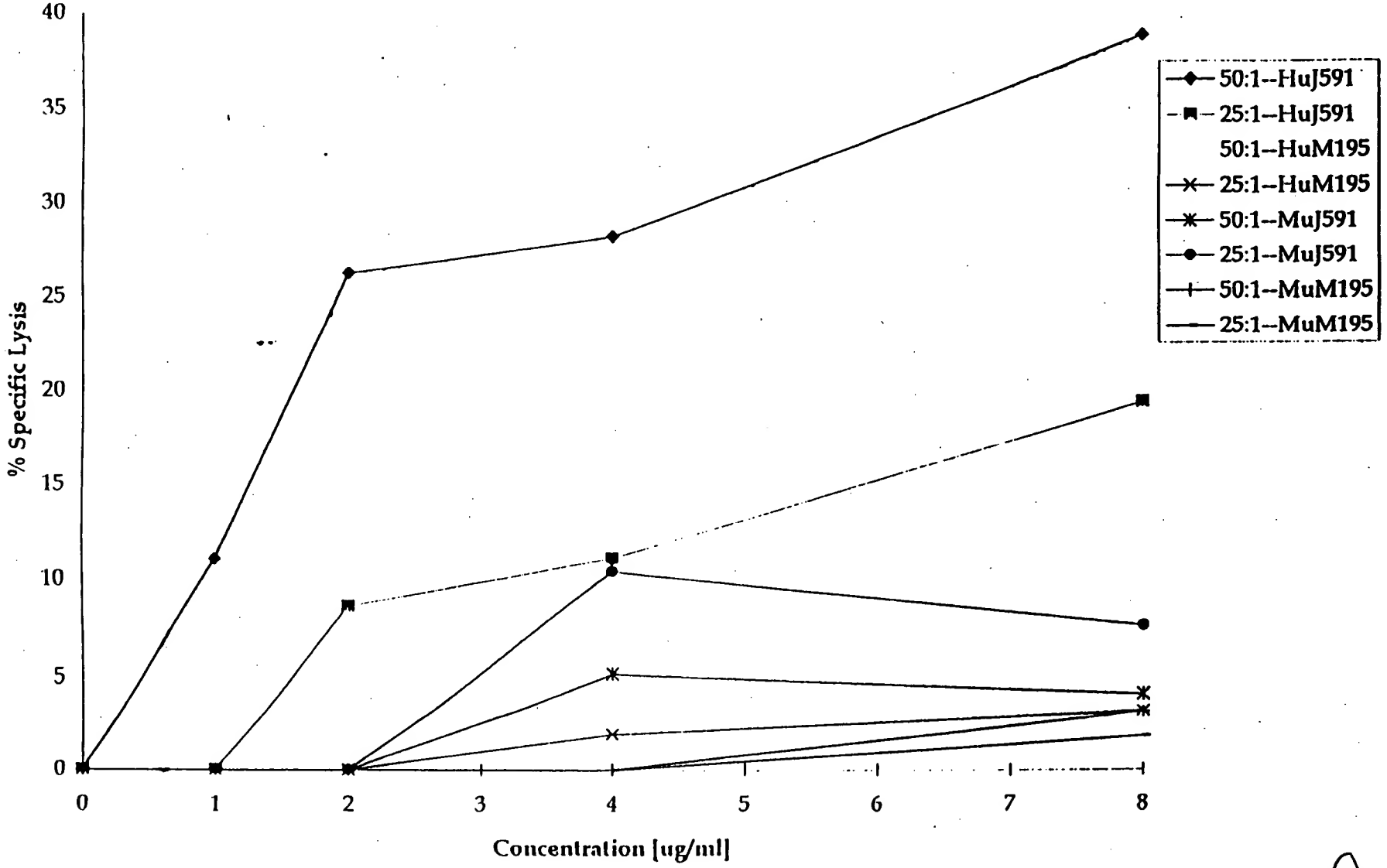



Fig. 4

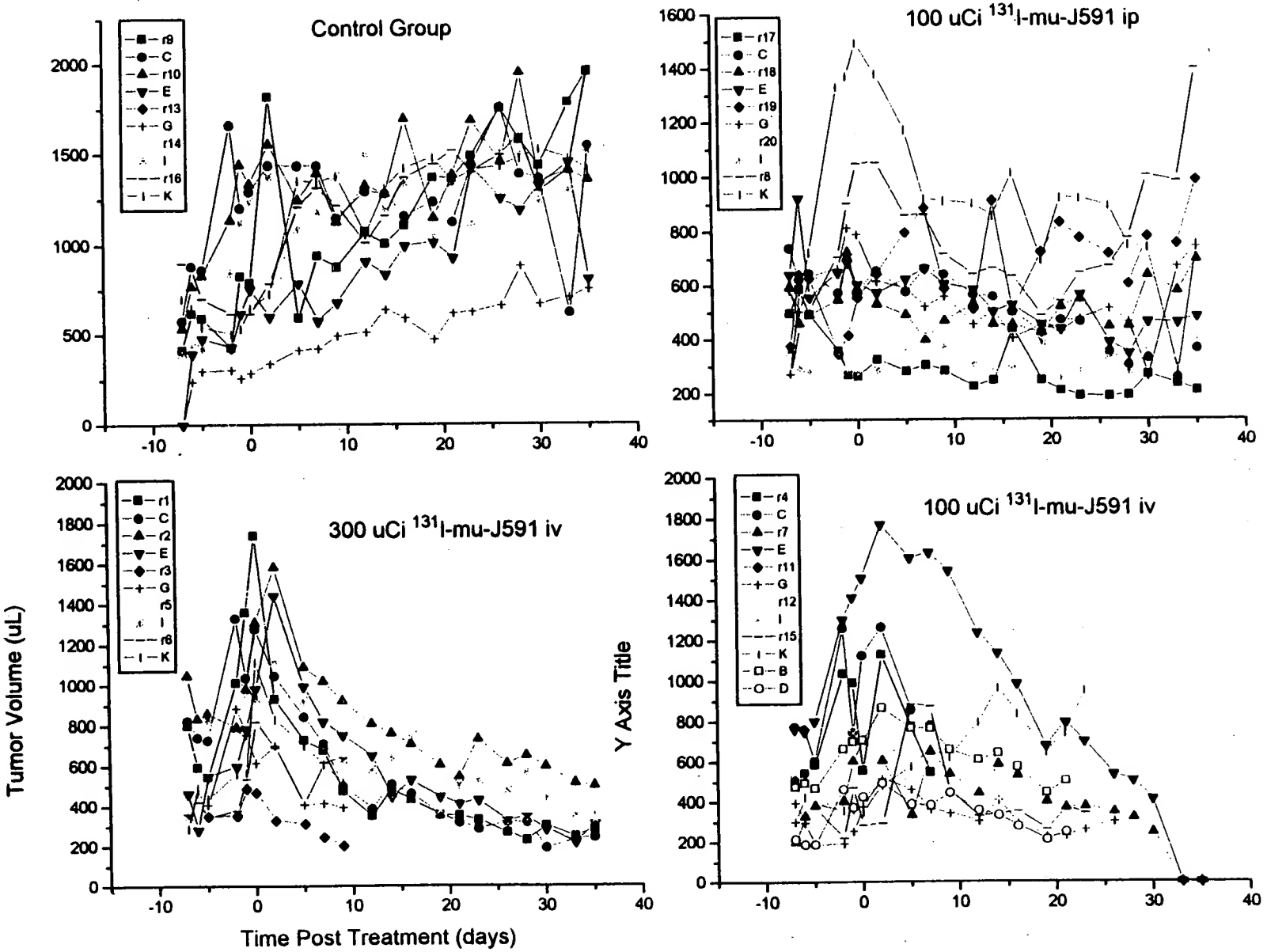
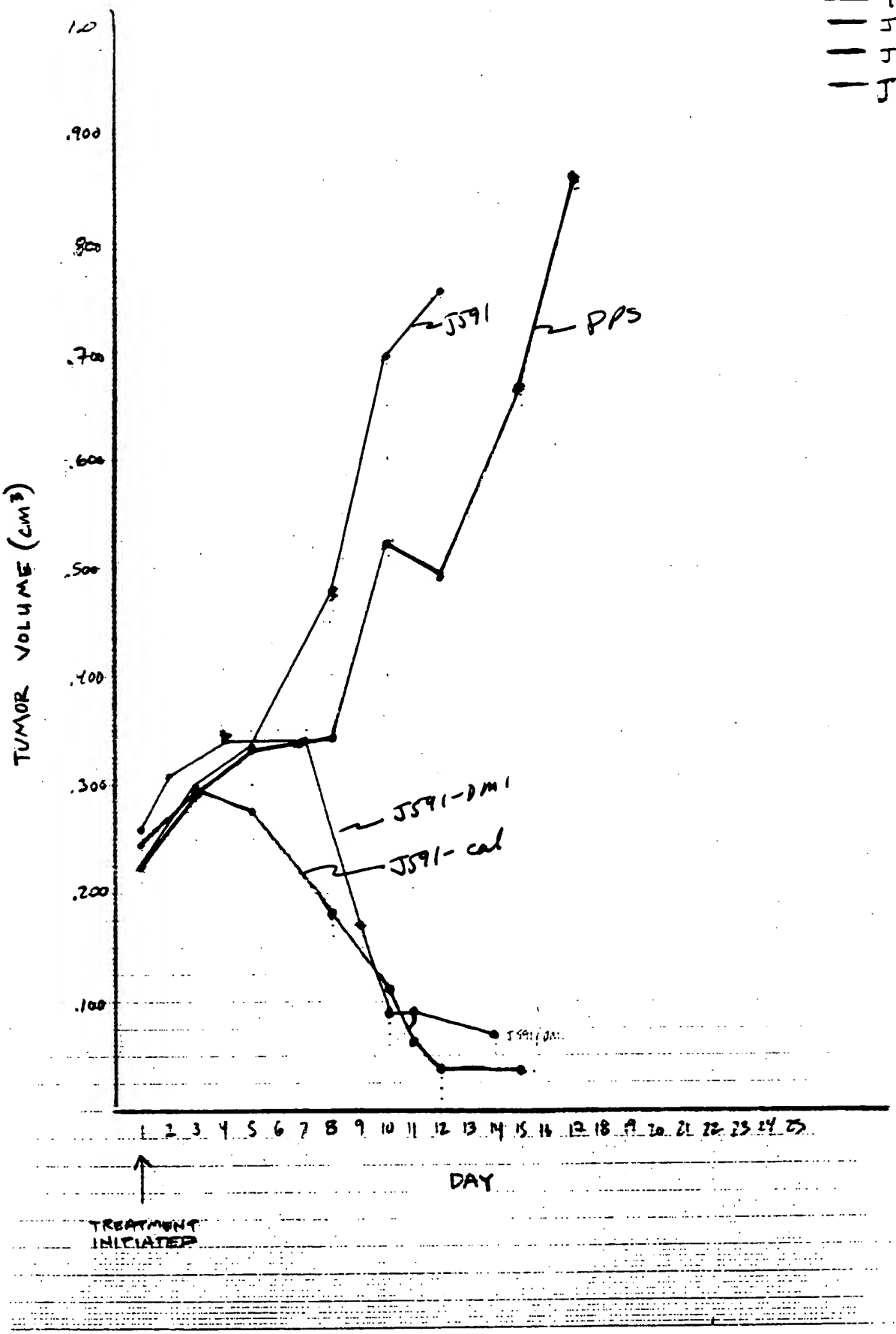



Fig. G1

30 mg in A6

G2a




Millimeter to the Centimeter

Fig. G2a

G2b

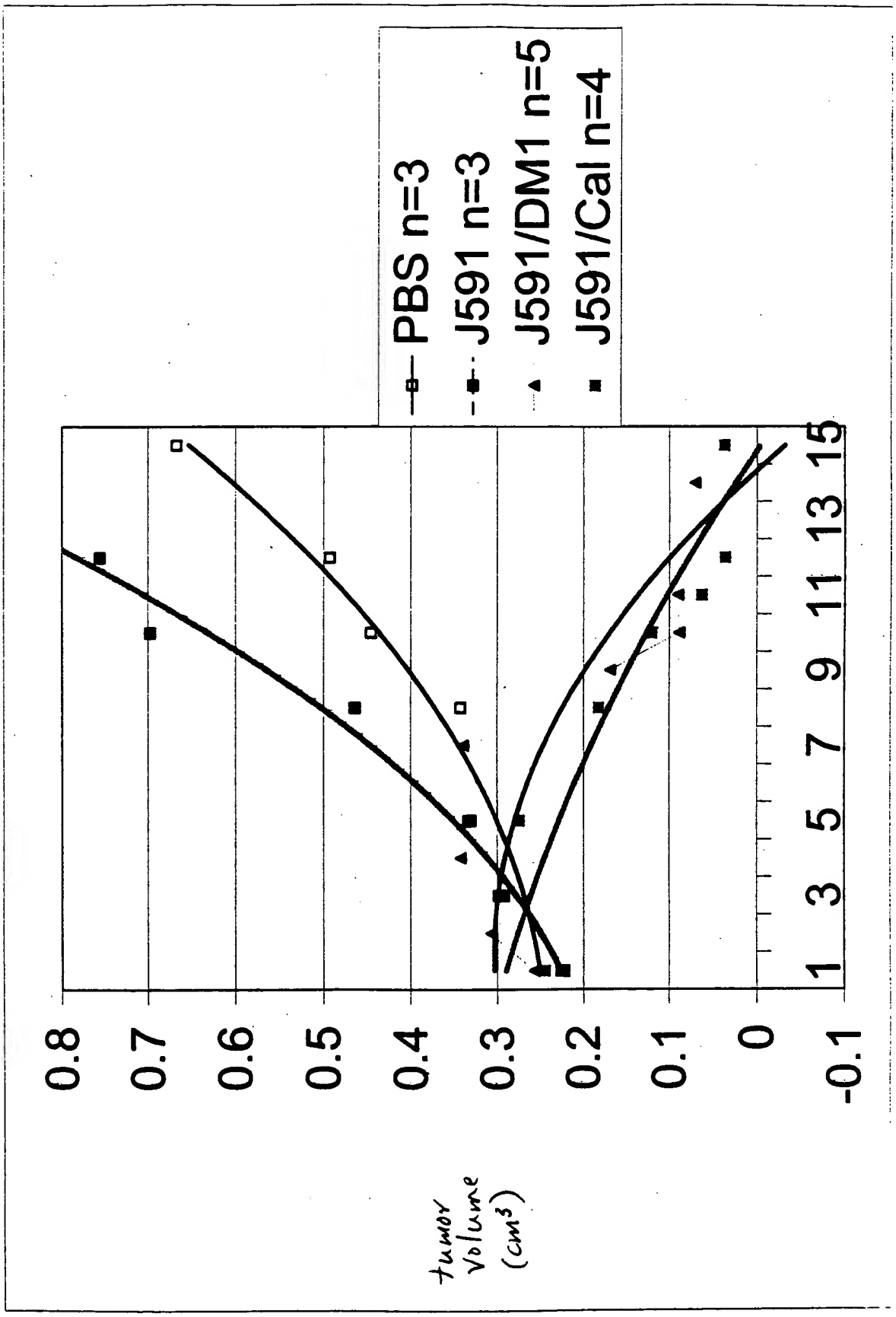

PBS n=3
J591 n=3
J591/DM1 n=5
J591/Cal n=4
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
1 3 5 7 9 11 13 15
tumor volume (cm³)

Fig. G2b

G2c

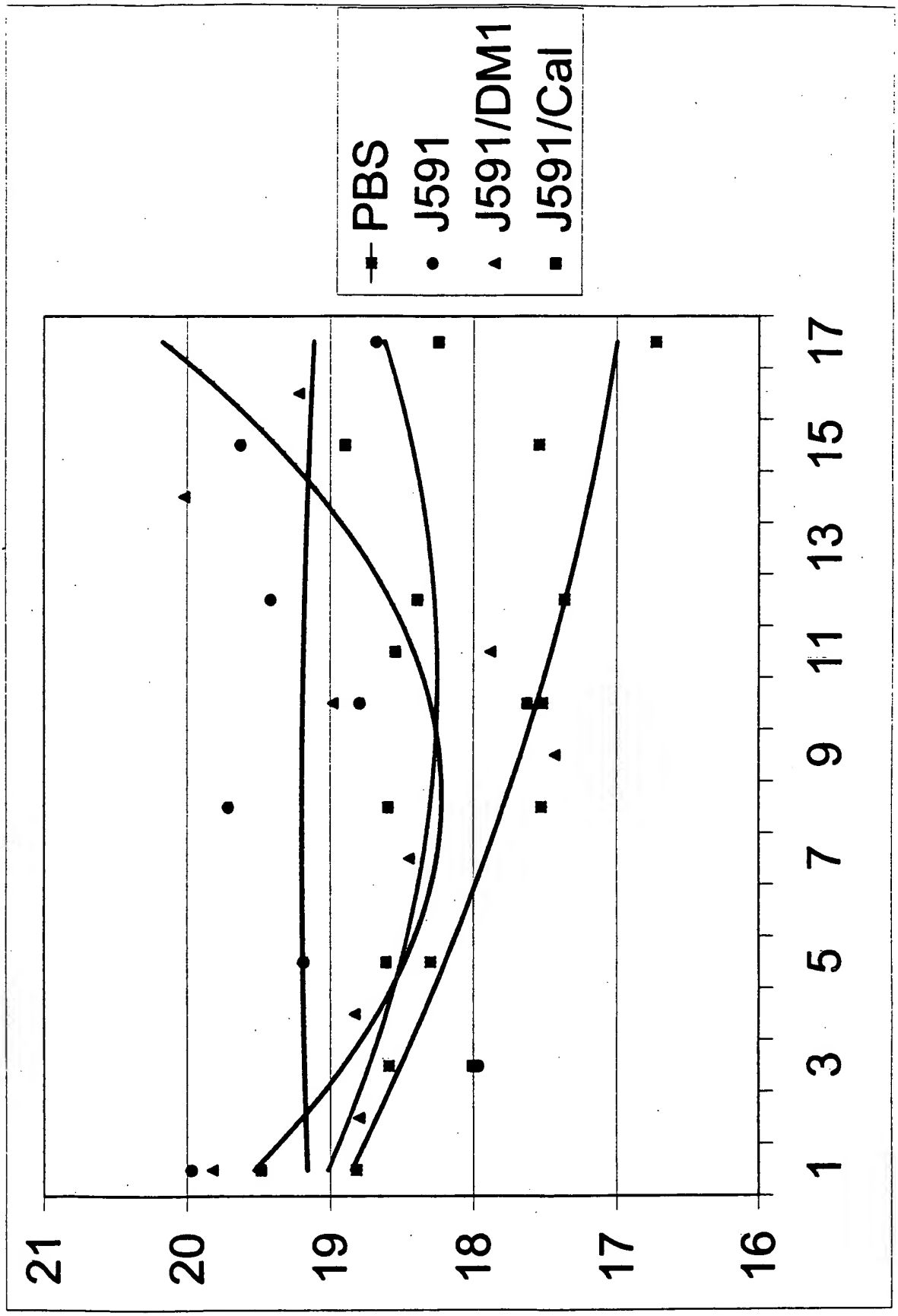



Fig. G2c

G3a

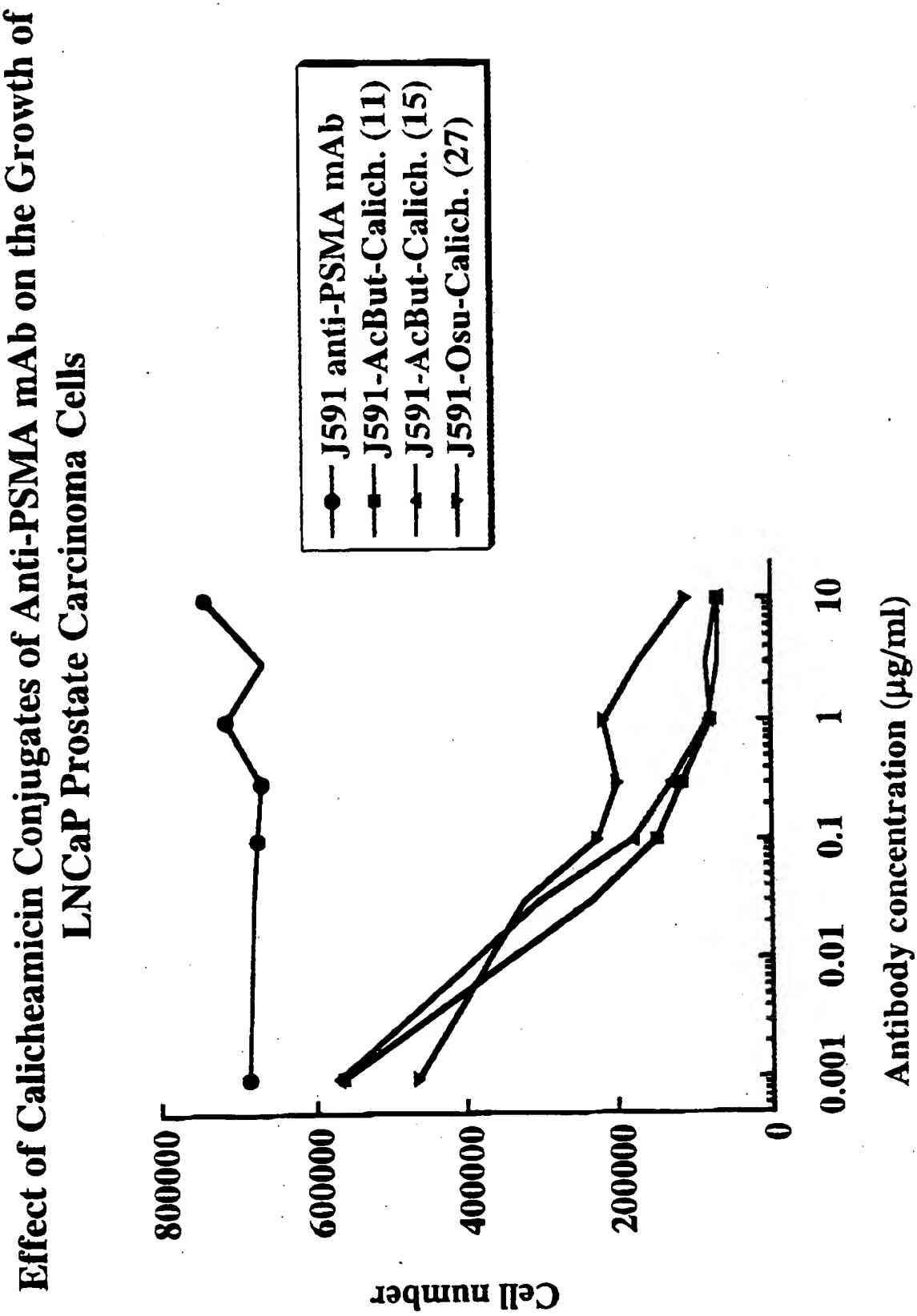

Effect of Calicheamicin Conjugates of Anti-PSMA mAb on the Growth of LNCaP Prostate Carcinoma Cells
J591 anti-PSMA mAb
J591-AcBut-Calich. (11)
J591-AcBut-Calich. (15)
J591-Osu-Calich. (27)
Cell number
800000
600000
400000
200000
0
0.001
0.01
0.1
1
10
Antibody concentration (µg/ml)

Fig. G3a

G36

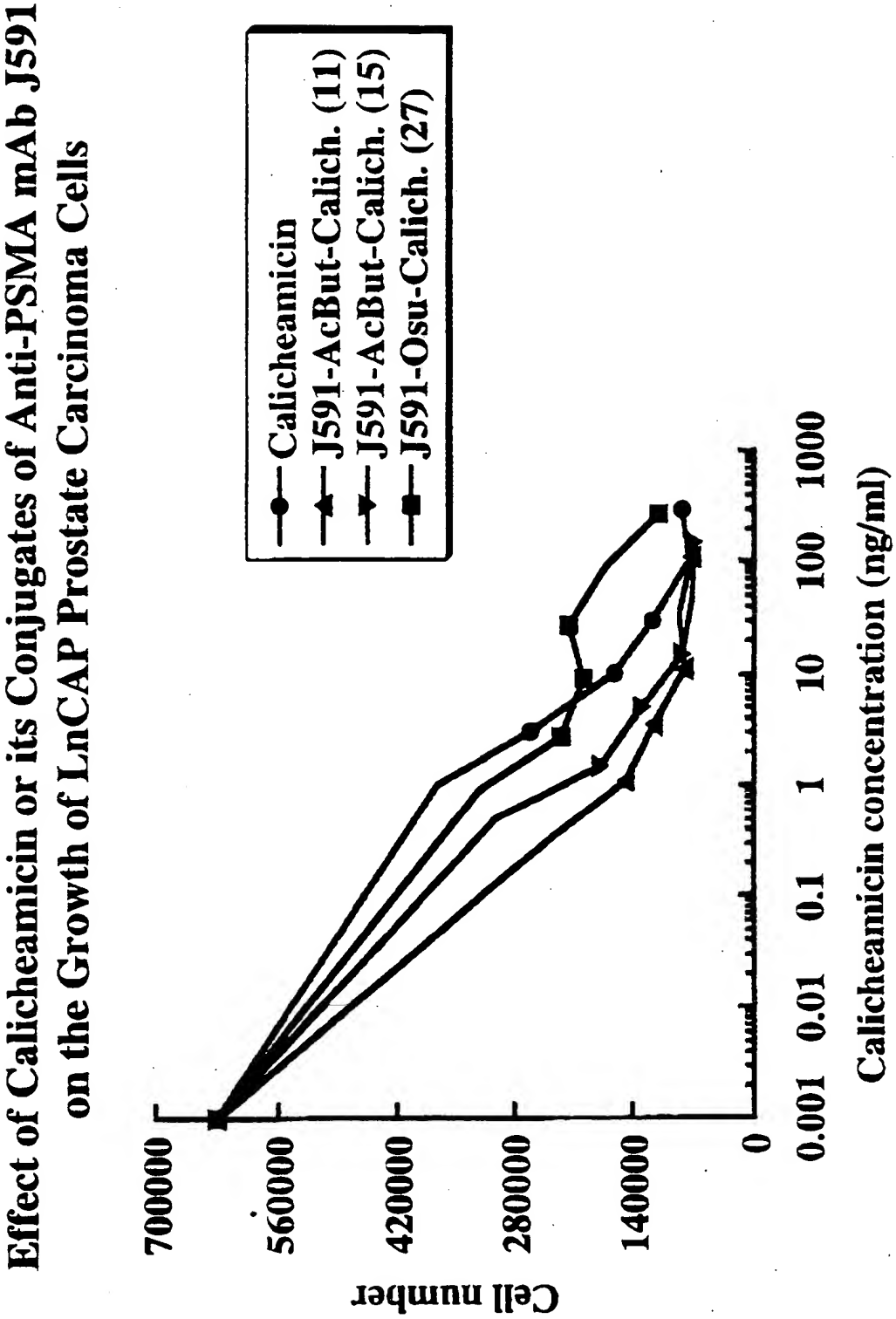

Effect of Calicheamicin or its Conjugates of Anti-PSMA mAb J591 on the Growth of LnCAP Prostate Carcinoma Cells
Calicheamicin
J591-AcBut-Calich. (11)
J591-AcBut-Calich. (15)
J591-Osu-Calich. (27)
Cell number
700000
560000
420000
280000
140000
0
0.001 0.01 0.1 1 10 100 1000
Calicheamicin concentration (ng/ml)

Fig. G3b

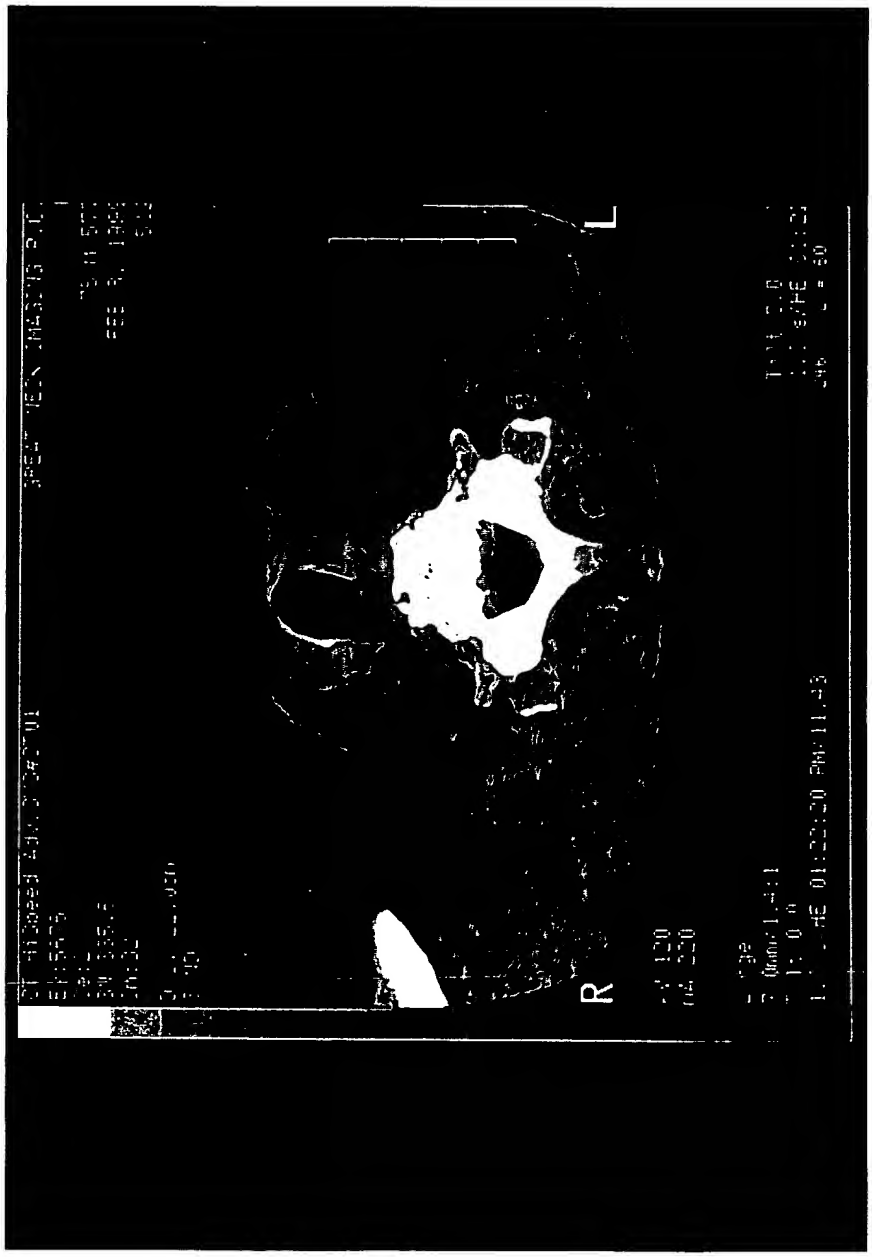



Fig. H

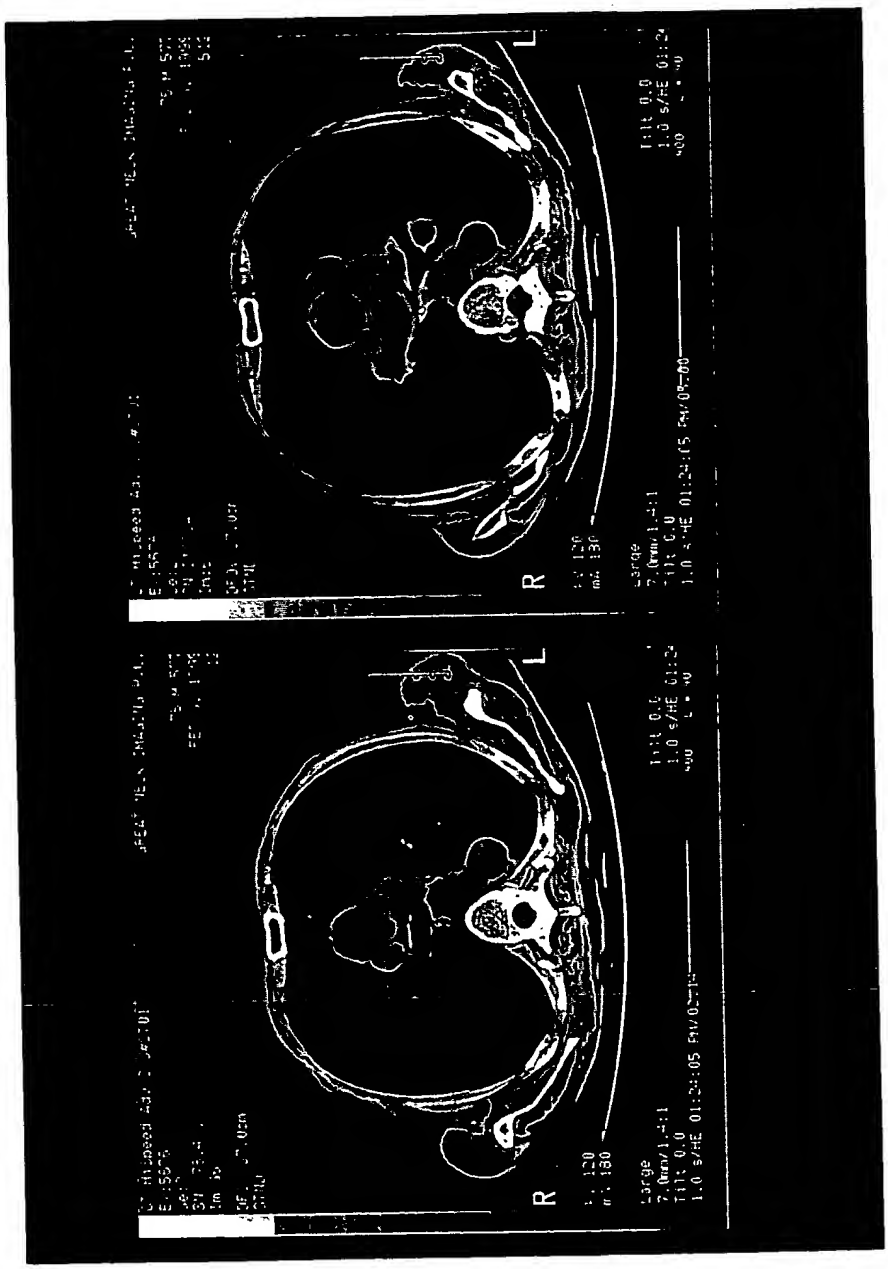



Fig. H

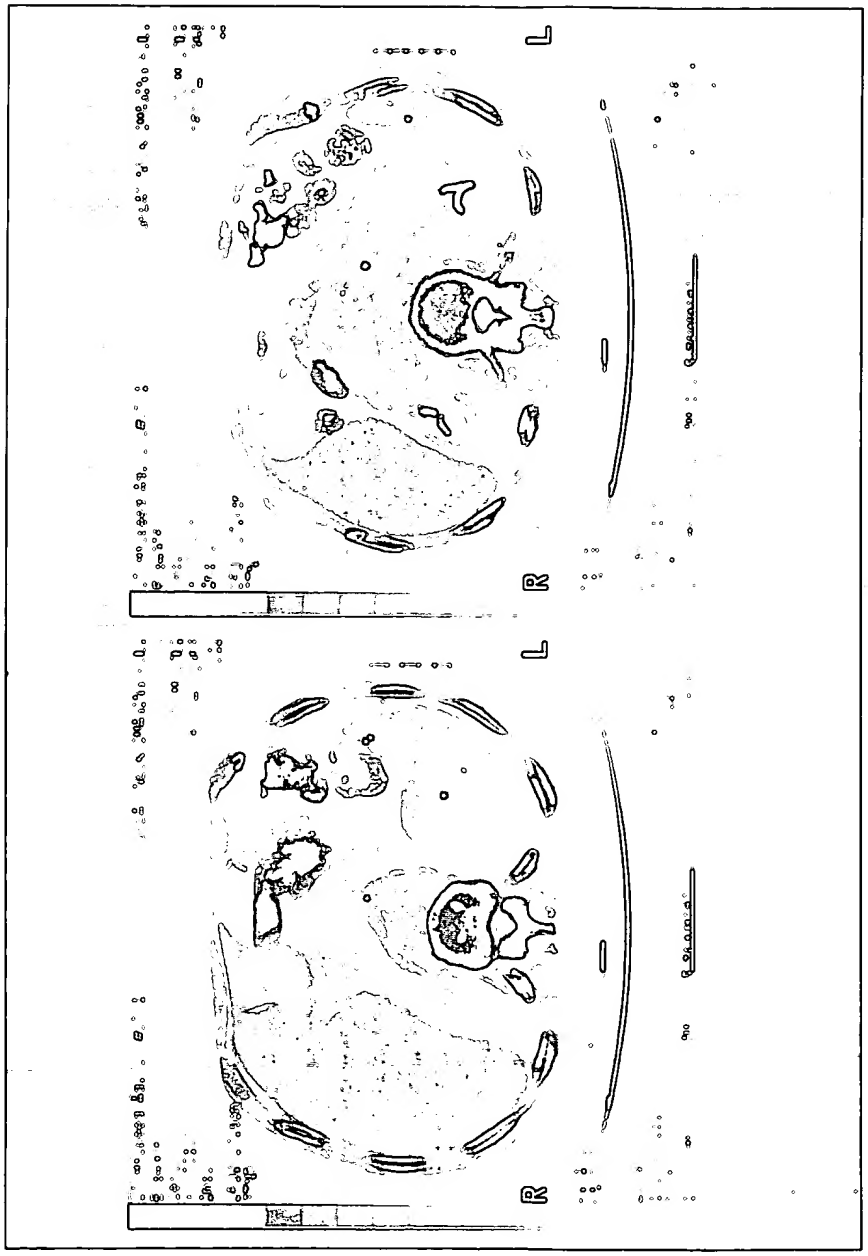



Fig. H

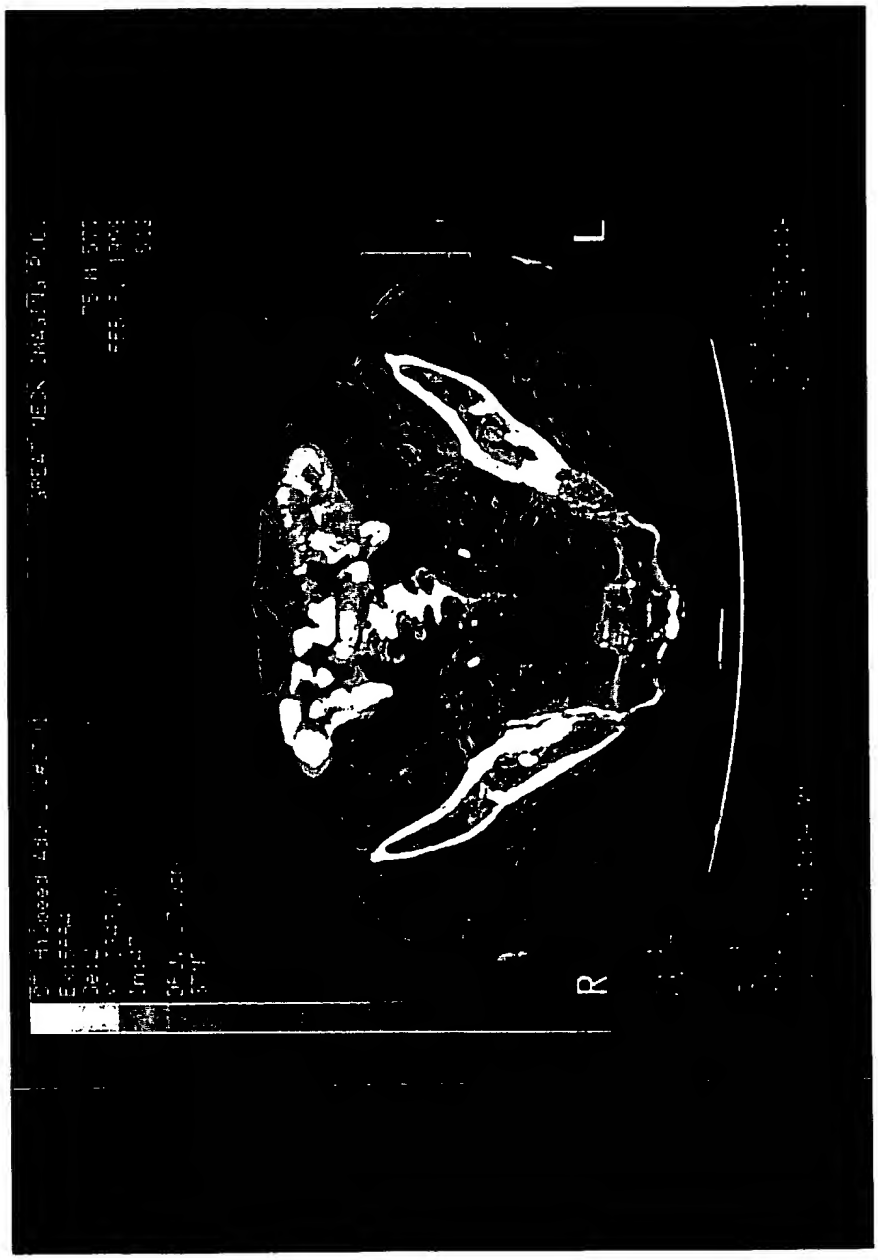



Fig. H

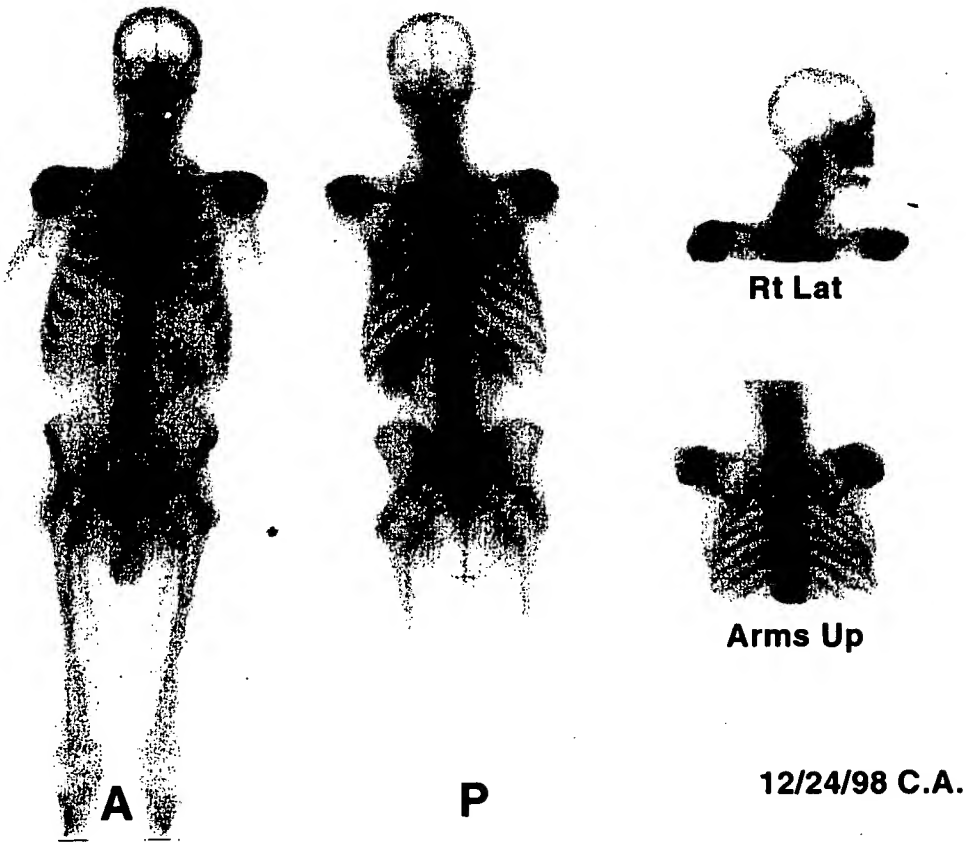



Fig. H

H1

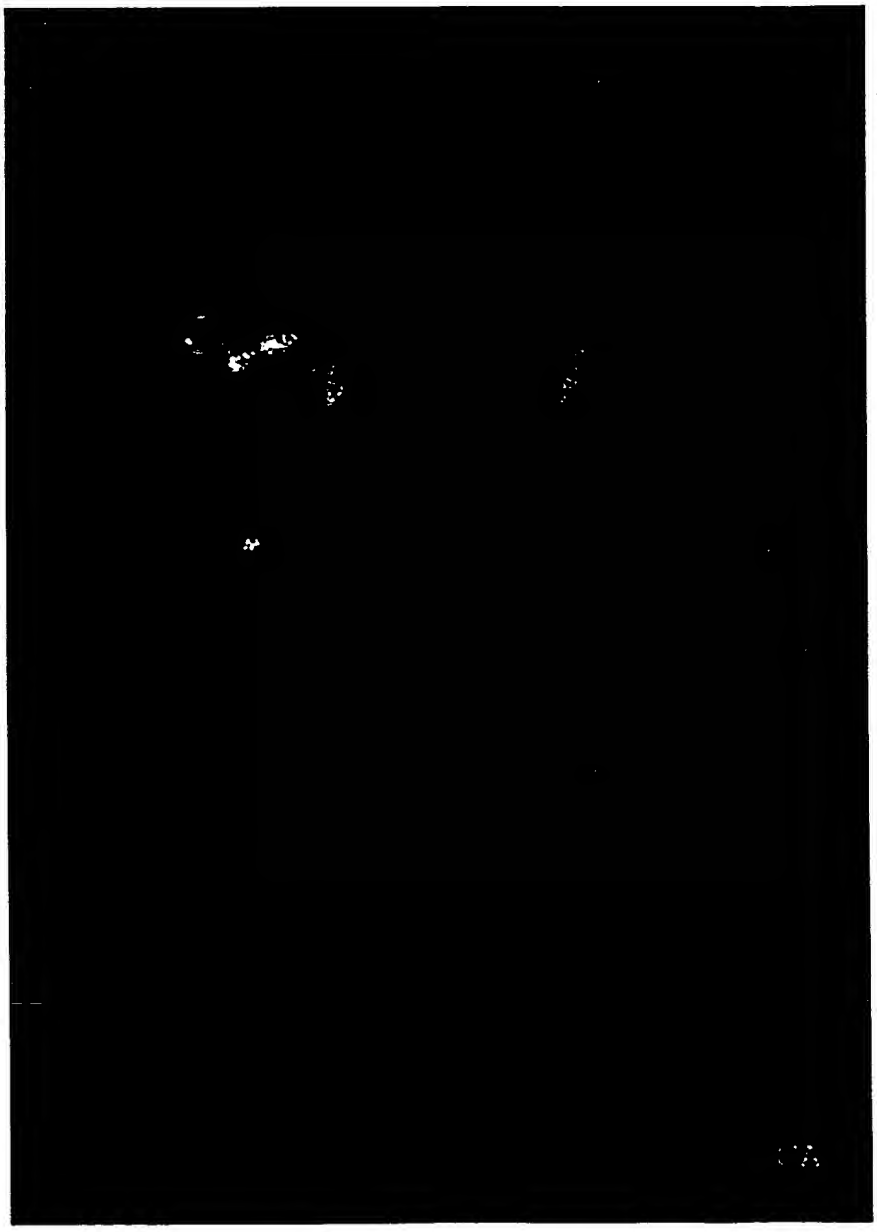


Fig. H1

H2

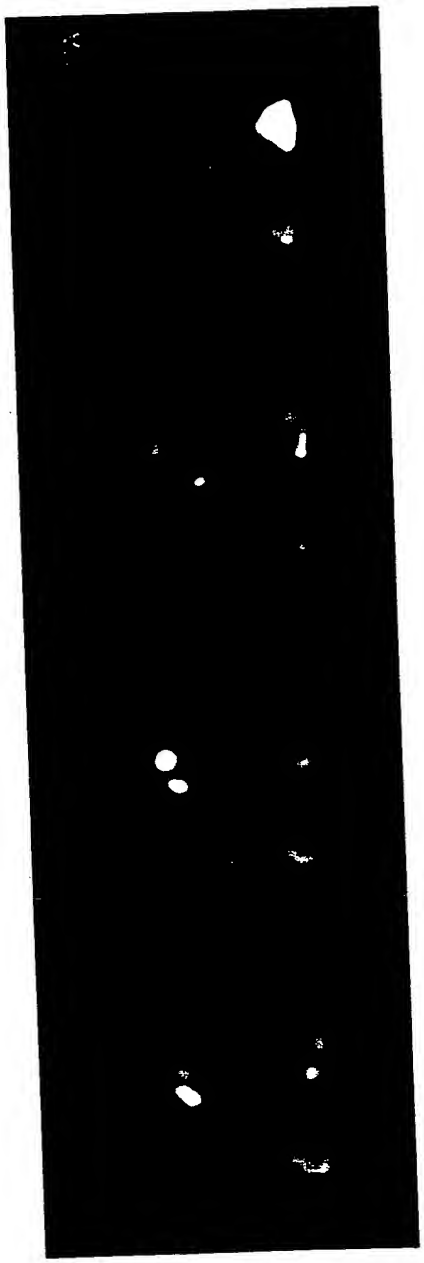


Fig. H2

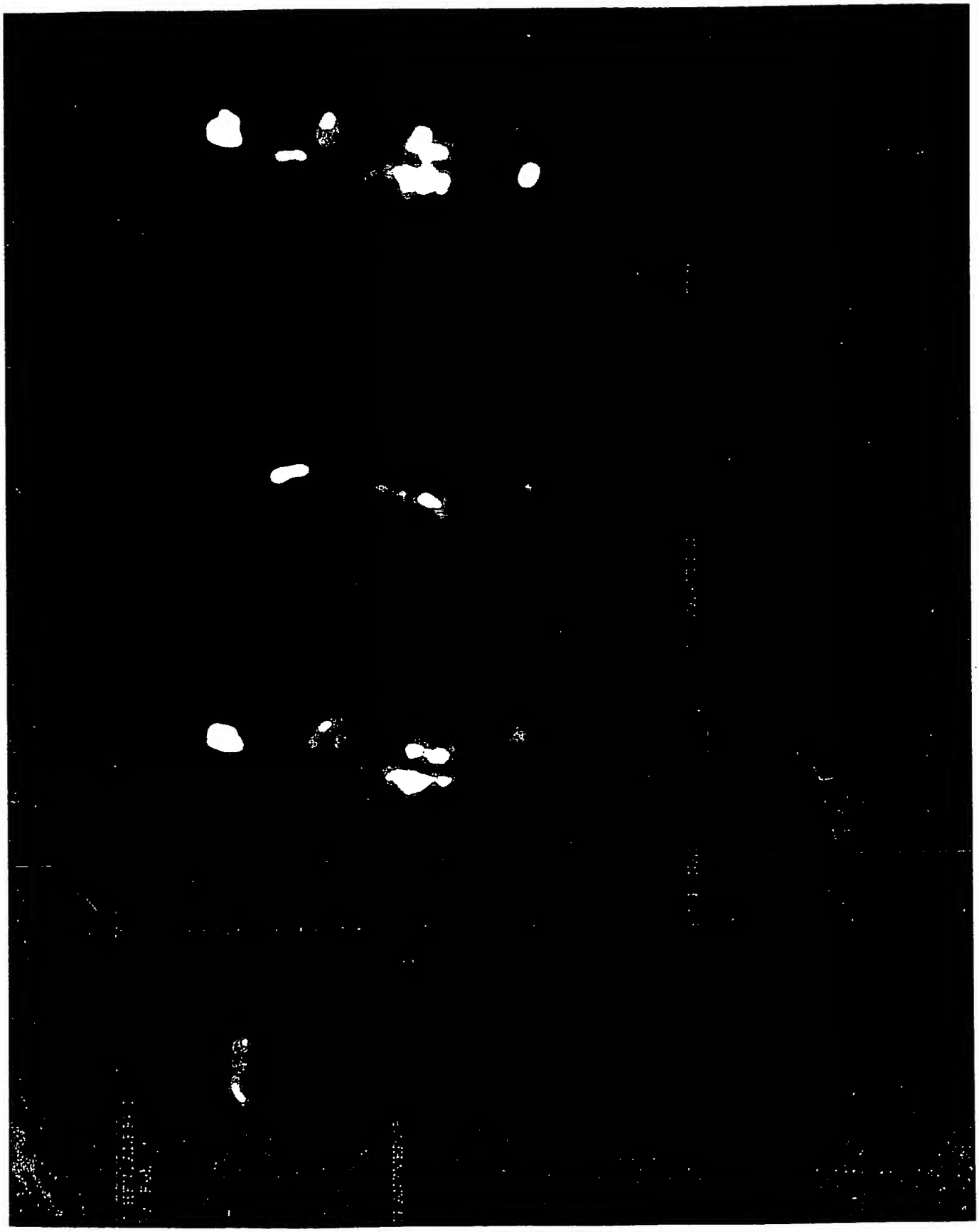


Fig. H

ELSEVIER

# Biochemical Characterization and Mapping of the 7E11-C5.3 Epitope of the Prostate-Specific Membrane Antigen

John K. Troyer, BS, Qi Feng, PhD, Mary Lou Beckett, MS, and George L. Wright, Jr, PhD

**The expression of the prostate-specific membrane antigen (PSMA) glycoprotein recognized by the murine monoclonal antibody (MAb) 7E11-C5.3 has been shown to be highly restricted to prostate epithelium. Although the conjugated form of this MAb (CYT-356) may soon be used clinically for in vivo imaging of extraprostatic disease, few details regarding the nature of the antigenic epitope of PSMA have been reported. This study was carried out to analyze the MAb 7E11-C5.3 epitope on PSMA using standard biochemical techniques, and the antigenic epitope was mapped with synthetic peptides. The MAb 7E11-C5.3 epitope was susceptible to both periodic acid oxidation and proteolytic digestion, which indicated that the antigen consisted of a glycoprotein. However, additional biochemical assays such as sodium borohydride, tunicamycin treatment, and digestion with glycosidases failed to abrogate MAb 7E11-C5.3 binding. Epitope mapping with synthetic peptides demonstrated the epitope to be localized to the intracellular domain at the N-terminus of the PSMA molecule with a minimal reactive peptide consisting of six amino acids (MWNLLH). The synthetic peptides were treated with periodic acid, which resulted in inhibition of antibody binding, suggesting that treatment of the PSMA antigen resulted in damage to the peptide chain. These data suggest that the MAb 7E11-C5.3 does not recognize a glycopeptide as was initially thought, but recognizes an intracellular epitope consisting of only the primary polypeptide chain. Further studies are needed to determine how CYT-356 is able to image tumors in vivo when the antigenic epitope is intracellular.** *(Urol Oncol 1995;1: 29–37)*

Prostate cancer now exceeds lung cancer as the most commonly diagnosed cancer and is the second leading cause of cancer death in American men.[1,2] It is estimated that by the year 2000, there will be a 37% increase in prostate cancer deaths and a 90% increase in prostate cancer diagnosis.[3] In 1994 alone, there will be an estimated 200,000 new cases reported.[1] Although an increase in prostate cancer diagnosis may result from improved public awareness and education as well as screening of at-risk individuals,[4–6] a significant challenge will be to maintain the quality of life of those individuals diagnosed with this disease and to identify those patients who are likely to present with recurrent or metastatic disease.

Much has been learned about prostate cancer in recent years, yet there are no effective therapeutic strategies for recurrent prostate carcinoma.[7] In most cases of recurrent disease, serum prostate-specific antigen (PSA) levels begin to rise before the presence of other evidence of disease as assessed by current diagnostic modalities. As a result, the assumption is made that recurrent disease is present but is not detectable by standard imaging techniques. The clinical use of monoclonal antibodies (MAbs) recognizing prostate-specific biomarkers may provide a significant advance by allowing earlier detection of metastatic foci and site-directed immunotherapeutic approaches in patients with recurrent disease. One such MAb that may prove useful in this regard is the murine MAb 7E11-C5.3, developed using membrane-enriched fractions of the LNCaP prostate carcinoma cell line as an immunogen.[8] The antigen recognized by MAb 7E11-C5.3 is an approximately 100-kD transmembrane glycoprotein[9] designated the prostate-specific membrane antigen (PSMA). Recent studies have resulted in the molecular identification and characterization of the gene encoding for this prostate-specific biomarker.[10] The $^{111}$indium-labeled conjugated form of MAb 7E11-C5.3 ($^{111}$indium CYT-356) has been used to localize LNCaP xenograft tumor growth in nude mice and metastatic prostate carcinoma in humans[11,12]; it has also been demonstrated to safely detect sites of prostatic carcinoma recurrence after radical prostatectomy.[13,14] Clinical trials are ongoing to demonstrate further the efficacy of the CYT-356 immunoconjugate for imaging metastatic prostate disease, and studies are underway to determine whether this MAb could be used for targeted therapy approaches.

Preliminary immunohistochemistry studies[7,15] have indicated that the MAb 7E11-C5.3 epitope may be intracellular, based on the localization of staining and the inability of MAb 7E11-C5.3 to stain living cells. However, CYT-356 is able to image living tumors in vivo, and initial biochemical analysis seemed to indicate that the antigenic epitope contained a significant carbohydrate moiety. These data are conflicting unless the intracellular carbohydrate moiety is protected by an intracellular membrane compartment, because it is unlikely that a carbohydrate would be stable in the cytoplasmic

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Supported in part by a grant from CYTOGEN Corporation, Princeton, New Jersey.

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Urol Oncol 1995;1:29–37

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SSDI 1078-1439(95)00004-2

environment. At the same time, an intracellular localization of the epitope would make it difficult for CYT-356 to image a living tumor mass in vivo. Because there is some promise that CYT-356 may be a useful tool in clinical practice, it is imperative that these questions regarding the antigenic epitope on PSMA be fully resolved. The purpose of the present study was to determine the physical and biochemical characteristics of the MAb 7E11-C5.3 epitope on this novel prostate-specific biomarker and to precisely map the location of the antigenic determinant. This study confirms that the epitope recognized by the native and immunoconjugated MAb 7E11-C5.3 is intracellular and consists of only the polypeptide chain, and further suggests the sensitivity of antibody-directed imaging; the therapeutic approaches and development of in vitro immunoassays could be enhanced by the production of second-generation antibodies to antigenic epitopes present in the extracellular domain of PSMA.

## Materials and Methods

### Cells and Reagents

LNCaP cells were obtained from the American Type Culture Collection and grown in RPMI 1640 supplemented with L-glutamine, gentamicin (Sigma Chemical Co., St. Louis, MO), and 5% fetal calf serum (Gibco-BRL, Gaithersburg, MD). The monoclonal antibody MAb 7E11-C5.3, purified by protein-A affinity chromotography from murine ascites, was provided by Cytogen Corp. (Princeton, NJ). The MAb concentration was determined using a single radial immunodiffusion system (TAGO, Burlingame, CA). Lectins were obtained from E. Y. Laboratories (San Mateo, CA). O-glycanase and N-glycanase enzymes were purchased from Genzyme (Boston, MA). EDAC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide), OPD (0-phenylenediamine dihydrochloride), all other enzymes, carbohydrates, carbohydrate conjugates, and chemicals were purchased from Sigma Chemical Co. unless otherwise noted.

### Membrane Preparations

LNCaP cells were harvested and pelleted by centrifugation at 1000 × *g*. The pellet was washed once with ice-cold phosphate-buffered saline (PBS: 136 mmol/L NaCl, 1.7 mmol/L KCl, 8 mmol/L $Na_2HPO_4$, 1.5 mmol/L $KH_2PO_4$, 0.9 mmol/L $CaCl_2$, 0.5 mmol/L $MgCl_2$, pH 7.4) and pelleted again. The pellet was resuspended in hypotonic buffer (1 mmol/L $NaHCO_3$) containing a protease inhibitor cocktail (0.28 mmol/L antipain, 0.75 mmol/L pepstatin, 60 mmol/L ethylenediaminetetra-acetic acid) and incubated on ice for 30 minutes, then dounce homogenized. The homogenate was centrifuged at 2000 × *g* for 5 minutes in a Beckman JA20 rotor to pellet whole cells and nuclei. The supernatant was collected and centrifuged at 138,000 × *g* for 2 hours. The supernatant was discarded and the pellet, representing a crude membrane preparation, was resuspended in PBS and stored at −70°C.

### PSMA Affinity Purification

Membrane preparations were resuspended in solubilization buffer (30 mmol/L Tris, pH 7.5, 0.5 mol/L NaCl, 1% Nonidet P-40 [NP-40], 0.5% deoxycholate, 0.1% sodium dodecylsulfate [SDS], 0.5 mmol/L dithiothreitol [DTT]) and protease inhibitor cocktail (as described above) and incubated for 2 hours at 4°C on a rotator. The solubilized material was centrifuged at 100,000 × *g* for 1 hour at 4°C to pellet nonsolubilized material. The supernatant was collected, diluted 1:2 with 20 mmol/L Tris, pH 8.0, and loaded onto an Affinica Protein A-7E11-C5 affinity column, constructed using the manufacturer's instructions (Schleicher & Schuell, Keene, NH). The column was washed with wash buffer (20 mmol/L Tris, pH 8.0, 0.1% NP-40, 0.1 mmol/L DTT) and eluted with 2N $NH_4OH$, pH 11.0. The eluted fraction was placed in 3500-MW cutoff dialysis tubing (Spectrum, Houston, TX) and dialyzed against 20 mmol/L Tris HCl, pH 4.0, containing 0.1 mmol/L DTT for 2 hours at 4°C. The eluant was then concentrated against polyethylene glycol compound (MW 15,000–20,000) to approximately 500 μL. Protein concentrations were estimated using the bicinchonic acid (BCA) protein assay, following the manufacturer's instructions (Pierce, Rockford, IL).

### Physical and Biochemical Treatment

Purified PSMA from crude LNCaP membrane preparations was boiled for 10 minutes in the presence or absence of mercaptoethanol or SDS. Periodate oxidation[16] and sodium borohydride[17] treatments were carried out as described previously. Proteolytic digestion was achieved by incubating the purified PSMA at 37°C for 24 hours using 100 μL of the following protease solutions: trypsin type III (1, 10, and 100 U/mL), alpha-chymotrypsin type VII (5, 50, and 500 mU/mL), protease type XXI (2, 20, and 200 mU/mL), and protease type XXVI (8, 80, and 800 mU/mL). The treated PSMA was then analyzed using a modified radioimmunoassay (RIA).[18]

### Glycosidase Treatment

Purified PSMA was treated with beta-galactosidase, fucosidase, endo F, and chondroitinase ABC in Eppendorf tubes, following methods described previously.[19–21] For the N-glycanase digestion, the antigen was boiled for 3 minutes in the presence of 0.5% SDS and 0.1 mmol/L mercaptoethanol, then diluted in PBS containing 10 mmol/L phenanthroline and NP-40. The N-glycanase (Genzyme, Boston, MA; 0.3 mU) was added and the reaction incubated overnight at 37°C. For the O-glycanase digestion, the antigen was first denatured in SDS and mercaptoethanol as for the N-glycanase treatment, then digested with neuraminidase for 2 hours. O-glycanase (0.5 mU) was added and the reaction mixture incubated overnight at 37°C.

### Competitive Binding Experiments

The carbohydrate concentrations were adjusted to 0.05 mmol/L with PBS. The 7E11-C5.3 MAb was incubated with the carbohydrate or PBS control for 2 hours at room temperature. Fifty microliters of the mixture was then used as the primary antibody for an RIA using purified PSMA from LNCaP membrane extract as the antigen. For lectin competitive binding studies, lectins were used at a concentration of 1 mg/mL in PBS. The lectins or PBS control was added to antigen-coated wells in a volume of 100 μL and incubated for 2 hours at room temperature. The wells were washed three times with PBS,

and an RIA was performed as described above. Data for both the carbohydrate and lectin experiments were expressed as a percentage of the control binding using the following formula:

$$\% \text{ control binding} = \frac{\text{CPM of treated well}}{\text{CPM of PBS control well}} \times 100,$$

in which cpm = counts per minute.

## Tunicamycin Treatment

LNCaP cells were cultured in the presence of tunicamycin for 7 days, as described previously.[22] The cells were harvested and membrane preparations were prepared as described above.

## Peptide Synthesis

Peptides were synthesized on a Synergy Peptide Synthesizer (Perkin Elmer-Applied Biosystems, Foster City, CA). Cleavage and extraction of the crude peptide were performed according to the manufacturer's instructions. All peptides gave a single discrete peak when analyzed by reverse-phase chromatography on a Waters 650 high-performance liquid chromatography system. The sequence of the peptides was derived from the published nucleotide sequence of PSMA[10] and named according to the distance from the N-terminus of the deduced amino acid sequence (Table 1).

## Direct Binding Peptide Assay

Peptides were bound to wells of microtiter plates using the procedure described previously,[23] with several modifications. Briefly, 100 μL of bovine serum albumin (BSA) (1 μg/mL) was dispensed into each well of a high-binding enzyme immunoassay (EIA) plate (Costar, Cambridge, MA) and dried overnight. The plates were rinsed twice with PBS, followed by two rinses with distilled water. Various concentrations of peptides were added to the wells in a volume of 50 μL, followed by 50 μL of EDAC cross-linker (10 mg/mL) or water control, and the plates were incubated overnight at 4°C with shaking. The plates were washed five times with wash buffer (PBS containing 0.05% Tween-20). The concentration of peptides adhered to the plate was determined by protein assay of the peptide-EDAC mixture before and after the cross-linking incubations. A standard enzyme-linked immunosorbent assay (ELISA) was then performed (as described below).

## Competitive Binding Peptide Assay

Competition plates were made by first dispensing 100 μL BSA (1 μg/mL) into the wells of a high-binding EIA plate and drying overnight. The plate was rinsed twice with PBS and twice with distilled water before the addition of peptides. Serial 1:5 dilutions of each peptide were made with water, starting at a concentration of 1 mmol/L and with a final volume of 40 μL in each well. The antibody concentration used was determined by finding the concentration of MAb 7E11-C5.3 that gave half maximal binding to a constant concentration of purified PSMA antigen at 200 ηg per well, determined to be 1 μg/mL. Forty microliters of MAb 7E11-C5.3 (1 μg/mL) was added to each well, and the plate was incubated at 4°C overnight with shaking.

Antigen plates were prepared by dispensing 100 μL of purified PSMA (2.0 μg/mL) into the wells of high-binding EIA plate (Costar) and incubating overnight at 4°C with shaking. The antigen plates were washed four times with wash buffer and blocked with blocking buffer (1% BSA in PBS containing

**TABLE 1.** AMINO ACID SEQUENCES OF SYNTHETIC PSMA PEPTIDES

| Name[a] | Amino acid sequence |
|---|---|
| N1.19 | M W N L L H E T D S A V A T A R R P R |
| N1.12 | M W N L L H E T D S A V |
| N1.11 | M W N L L H E T D S A |
| N1.10 | M W N L L H E T D S |
| N1.9 | M W N L L H E T D |
| N1.8 | M W N L L H E T |
| N1.7 | M W N L L H E |
| N1.6 | M W N L L H |
| N1.5 | M W N L L |
| N1.4 | M W N L |
| N2.6 | W N L L H |
| N7.12 | E T D S A V |
| N13.19 | A T A R R P R |
| NΔ1.6 | C W N L L H |
| N1.6Δ | M W N L L Y |
| N344 | M H I H S T |
| N470 | M Y S L V H |
| N583 | M V F E L A |
| N664 | M N D Q L M |
| N669 | M F L E R A |

[a]Named according to the distance from the N-terminus of the PSMA amino acid sequence.

0.05% Tween-20) for 1 hour at room temperature. The blocking buffer was removed, and the 80 μL of the peptide-antibody mixture from the inhibition plates prepared above was transferred to the antigen plates and used as the primary antibody for a standard ELISA.

### Standard ELISA

Microtiter plates, activated with peptide or antigen, were blocked for 1 hour at room temperature with blocking buffer (1% BSA in PBS containing 0.05% Tween-20) with shaking. The blocking buffer was removed, and MAb 7E11-C5.3 or competition mix (as described above) was added to each well and incubated for 2 hours at room temperature with shaking. The plates were washed five times with wash buffer, with the final wash being removed by vacuum aspiration to ensure that all of the wash buffer was removed. The secondary antibody (1 μg/mL horseradish peroxidase-labeled, horse anti-mouse antibody; Vector Laboratories, Burlingame, CA) was added at 50 μL per well and incubated for 2 hours at room temperature with shaking. The plates were washed five times with wash buffer, and all of the buffer was removed with the final wash. After the final wash, 200 μL of OPD substrate was added to each well and incubated for 5–30 minutes with shaking, and read at 405 nm on an EL-340 Microplate reader (Bio-Tek Instruments, Winooski, VT).

### Determination of Affinity Constant ($K_a$)

The affinity of MAb 7E11-C5.3 for the native antigen and peptides was determined by a modification of a method described previously.[24] The MAb binding was plotted as bound/free antibody as a function of the concentration of bound antibody (mmol/L). The regression coefficient ($K_a$) was calculated according to the method of Scatchard,[25] and the Student t test was applied to determine the significance of the results.

## Results

### Basic Biochemical Characterization of the PSMA Epitope

The initial characterization of the PSMA epitope used standard physical and biochemical techniques on purified material. The antigen was stable after incubations at 100°C and after reduction or denaturation in mercaptoethanol and SDS (Figure 1A). The epitope was susceptible to oxidation of vicinyl hydroxyl groups by periodic acid, suggesting a carbohydrate component of the epitope, although sodium borohydride (Figure 1A) and tunicamycin treatments (data not shown) were unable to inhibit binding. The increased binding of MAb 7E11-C5.3 to PSMA after sodium borohydride treatment most likely results from the release of 0-linked carbohydrates, which may mask the antigenic determinant.

The recognition of the PSMA epitope by MAb 7E11-C5.3 under reducing and denaturing conditions indicates that the integrity of the epitope was not dependent on the secondary or tertiary structure of the native molecule. Treatment of PSMA with a variety f proteases having differing specificities revealed the epitope to be highly susceptible to proteolytic digesti n (Figure 1B).

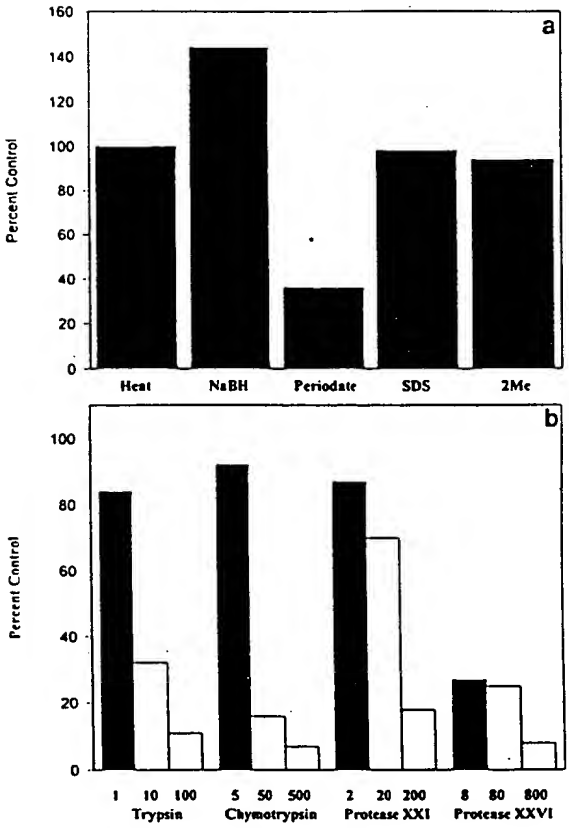


**FIGURE 1.** Basic physical and biochemical analysis of the PSMA epitope. A) Treatment of purified PSMA from LNCaP membrane extracts with heat (100°C for 10 minutes), sodium borohydride (NaBH), periodate, sodium dodecyl sulfate (SDS), and mercaptoethanol (2Me). The antigen was recognized in a denatured and reduced form and was susceptible to periodate oxidation but not sodium borohydride treatment. B) PSMA from LNCaP membrane extracts was highly susceptible to proteolytic digestion with trypsin (U/mL), chymotrypsin (mU/mL), protease type XXI (mU/mL), and protease type XXVI (mU/mL).

### Carbohydrate Analysis

To determine whether the antigenic epitope contained a carbohydrate moiety, we performed lectin inhibition studies. These experiments demonstrated that SBA, PNA, and MPA lectins, which are specific for D-galactose in either a monomeric form or a polymeric form, were able to reduce MAb 7E11-C5.3 binding, whereas lectins that bind to other carbohydrates such as mannose, glucose, fucose, neuraminic acid, and N-acetyl-glucosamine had no effect (Figure 2A). To prove the specificity of the lectin experiments, we used both general and specific glycosidases to digest PSMA. Neither O-glycosidase nor N-glycosidase, used to cleave O-linked and N-linked oligosaccharides, respectively, were able to reduce MAb 7E11-C5.3 binding. Digestion with specific enzymes, in particular beta-galactosidase, also did not inhibit antibody binding (Figure 2B). Similarly, MAb 7E11-C5.3 binding was not inhibited in competitive blocking experiments using specific car-

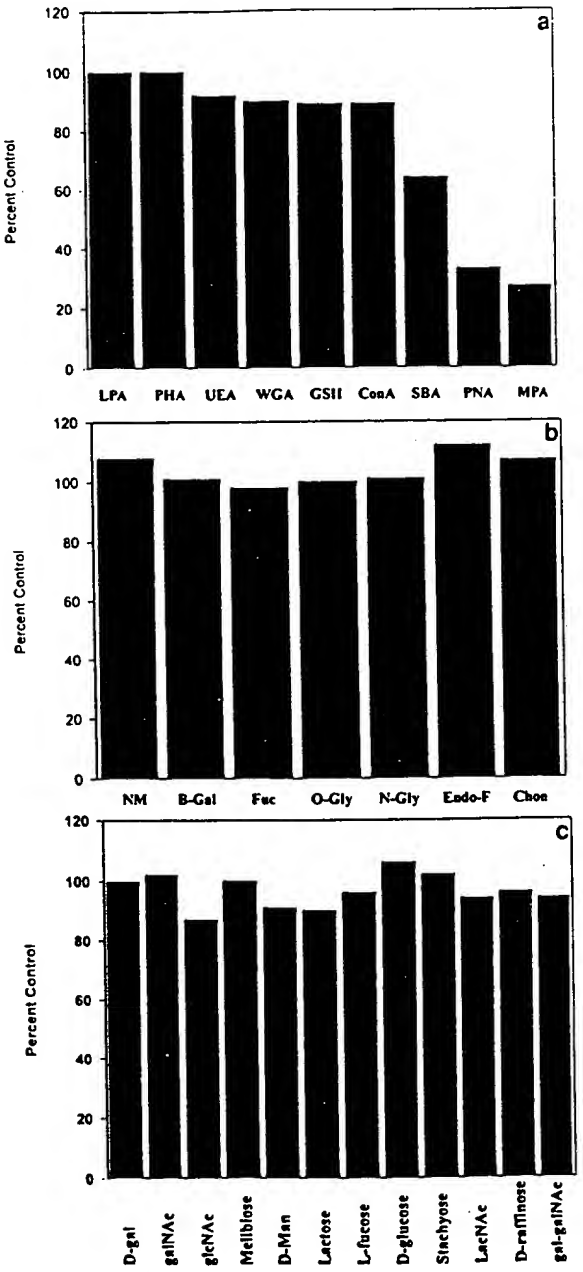


**FIGURE 2.** Carbohydrate analysis of the PSMA epitope. A) Competitive lectin-binding experiments showing inhibition with SBA, PNA, and MPA lectins. The carbohydrate specificities for the lectins are as follows: LPA (NeuNAc, D-galNAc, and D-GlcNAc), PHA (oligosaccharides), UEA (α-L-fuc), WGA (D-glcNAc, NeuNAc), GSII (D-glcNAc), ConA (α-D-man, α-D-glc), SBA (α-D-galNAc, D-gal), PNA (D-gal-β-(1-3)-D-galNAc), and MPA (α-D-gal). B) Digestion of PSMA from LNCaP membrane extracts with neuraminidase (NM), β-galactosidase (B-Gal), fucosidase (Fuc), O-glycanase (O-Gly), N-glycanase (N-Gly)), Endo F, and chondroitinase ABC (Chon). C) Competitive blocking with specific carbohydrates and aminosugars, demonstrating the inability to block MAb 7E11-C5.3.

bohydrates or aminosugars (Figure 2C). In particular, D-galactose (D-gal) and N-acetyl galactosamine (galNAc) were unable to inhibit binding. These saccharides correspond to the specificities of the SBA, PNA, and MPA lectins, which were able to reduce binding. The inability of these sugars and glycosidases to abrogate binding indicates that the lectin inhibition may be nonspecific.

## Peptide Binding

Because anecdotal evidence suggested that the PSMA epitope was intracellular, several synthetic peptides were synthesized to correspond to the 19 amino acids of the proposed intracellular domain of PSMA. Peptide N1.19 consisted of the entire 19-amino acid sequence (MWNLLHETDSAVATARRPR), whereas peptides N1.6 (MWNLLH), N7.12 (ETDSAV), and N13.19 (ATARRPR) were synthesized to correspond to three segments of N1.19. Figure 3 shows that N1.19 and the N-terminal N1.6 peptides were recognized by MAb 7E11-C5.3, although N1.6 had approximately 30% less binding than N1.19. N7.12, N13.19, and N669 (MFLERA), a negative control peptide, were inactive, indicating that the PSMA epitope was predominantly composed of the core peptide structure and located at the amino terminal end of the glycoprotein.

## Epitope Mapping

To characterize further the peptide portion of the PSMA epitope, we mapped the intracellular epitope completely. Ta-

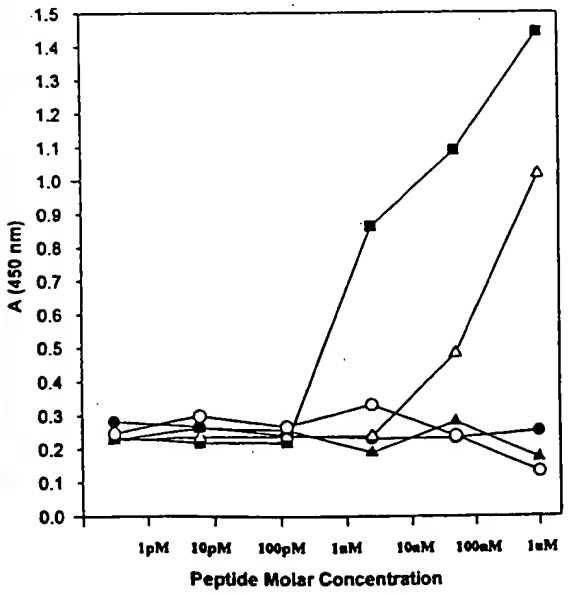


**FIGURE 3.** Direct-binding PSMA peptide assay with 50 μL of each molar concentration of peptide. Peptides were bound to EIA plates with BSA and EDAC chemical cross-linker and used for a standard ELISA with MAb 7E11-C5.3. The concentration of MAb 7E11-C5.3 used (1 μg/mL) was determined analytically from the half-maximal binding to the purified antigen. Peptides N1.19 (*solid squares*) and N1.6 (*open triangles*) were active in a dose-dependent manner, whereas peptides N7.12 (*closed circles*), N13–19 (*closed triangles*), and the control peptide N669 (*open circles*) were negative.

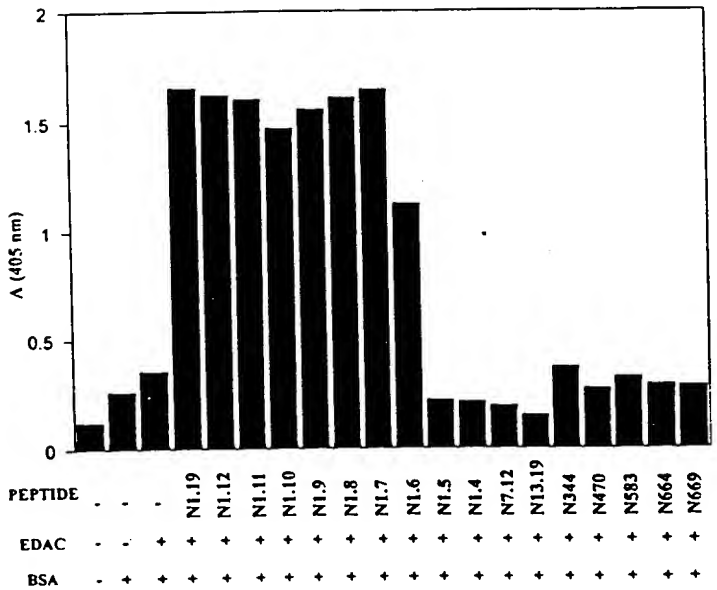


**FIGURE 4.** PSMA peptide mapping using 50 μL of each peptide at a concentration of 1 μmol/L. Baseline levels were defined by control wells containing only bovine serum albumin (BSA) or 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDAC), or both. All peptides containing at least the first seven amino acids were equally active, with N1.6 activity dropping by approximately one third. The methionine-containing negative control peptide N669 was below baseline, as were additional peptides from the PSMA amino acid sequence, which had a motif similar to N1.6.

ble 1 shows the orientation of the synthetic peptides, starting with the complete N1.19 peptide. Equimolar amounts of each peptide were assayed in a direct binding assay. All of the peptides containing the first seven amino acids had equal activity (Figure 4), whereas N1.6 retained approximately two thirds the activity and N1.5 was below baseline levels. These results indicate that the minimal reactive peptide was a 6-mer composed of the first six amino terminal amino acids (MWNLLH). Several additional peptides within the proposed PSMA amino acid sequence had a motif similar to N1.6, including N344, N470, N583, and N664. These peptides were also synthesized and analyzed, but were not bound by MAb 7E11-C5.3 (Figure 4). To demonstrate the specificity of the synthetic peptides and to use an alternative method that did not require binding of the peptides to the microtiter plates, we performed an indirect assay to block antibody binding competitively. Peptides N1.19 and N1.6 were able to inhibit binding of MAb 7E11-C5.3 to PSMA in a dose-dependent manner, whereas control peptides and other peptides present in the PSMA sequence were unable to compete for binding (Figure 5).

## *Biochemical Analysis of the Antigenic Peptides*

To determine whether the decrease in antibody binding after periodate treatment of the purified PSMA in Figure 1 resulted from the oxidation of the peptide epitope and not a carbohydrate, the synthetic peptides were treated with periodate before binding to the EIA plate for the direct binding assay. Periodate treatment of peptides N1.19 and N1.6 abrogated MAb 7E11-C5.3 binding (Figure 6). In addition, to mimic the effects of periodate oxidation of the polypeptide chain, peptide N2.6 (WNLLH), with a deletion of the methionine residue, or NΔ1.6 (CWNLLH), with a substitution of cysteine for the methionine residue, as well as peptide N1.5 (MWNLL), with a deletion of the histidine residue, or N1.6Δ (MWNLLY), with a substitution of tyrosine for the histidine residue, were synthesized and assayed in a direct binding assay. The deletion

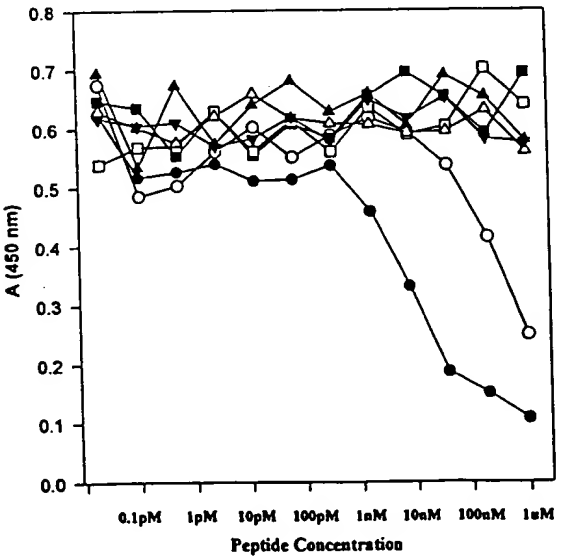


**FIGURE 5.** PSMA peptide inhibition assay. Forty microliters of each peptide dilution was incubated with 40 μL of the MAb 7E11-C5.3 and then used as the primary antiserum for an ELISA with purified PSMA from LNCaP cells as the antigen. Peptides N1.19 (*closed circles*) and N1.6 (*open circles*) competed in a dose-dependent manner, whereas the negative control peptide N669 (*open squares*) and peptides N334 (*upward triangles*), N470 (*downward triangles*), N583 (*closed squares*), and N664 (*open triangles*) were unable to block MAb 7E11-C5.3.

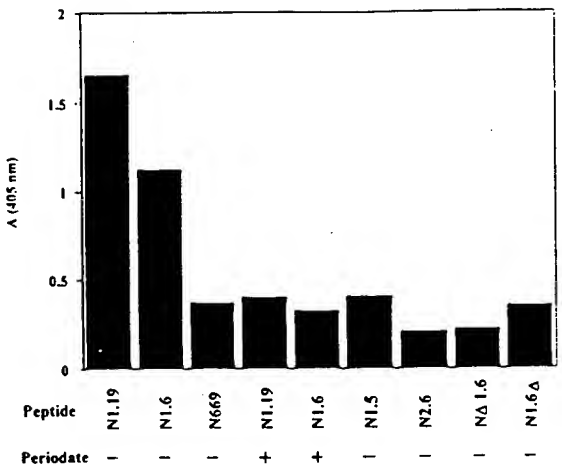


**FIGURE 6.** Analysis of the MAb 7E11-C5.3 epitope. Peptides N1.19 and N1.6 were treated with periodate as described for the native PSMA antigen. The activity of both peptides was reduced to the background levels of the negative control peptide (N669). In addition, deletion of the methionine (N2.6) and histidine (N1.5) or substitution of the methionine with cysteine (NΔ1.6) and substitution of the histidine with tyrosine (N1.6Δ) resulted in a similar loss of activity.

of either of these amino acids resulted in a loss of activity (Figure 6), suggesting that they or at least the amino acids present in that position are important for the structure of the epitope. Substitution of the methionine with cysteine and of the histidine with tyrosine also resulted in a loss of activity, indicating that the amino-terminal methionine and the carboxy-terminal histidine are required for structural integrity of the PSMA epitope and are essential for MAb 7E11-C5.3 activity, which, if lost by oxidation, would result in an abrogation of MAb 7E11-C5.3 binding.

The affinity of MAb 7E11-C5.3 was determined for the native PSMA glycoprotein and for the N1.19 and N1.6 active peptides. The affinity for the native PSMA glycoprotein ($K_a$ $1.16 \times 10^{10}$ $M^{-1}$) was approximately 6.8-fold higher than the affinity for the N1.19 peptide ($K_a$ $1.7 \times 10^9$ $M^{-1}$) and about 50-fold higher than that for the N1.6 peptide ($K_a$ $2.3 \times 10^8$ $M^{-1}$), although the affinity for N1.6 was only 7.4-fold lower than the affinity for N1.19.

## Discussion

The prostate-specific membrane antigen (PSMA) consists primarily of a major Mr 120,000 protein in tissue extracts and seminal plasma and an Mr 100,000 protein in LNCaP membrane extracts by Western blot analysis.[9,26,27] The amino acid sequence for the Mr 100,000 species expressed in the LNCaP cell line indicates that PSMA is a transmembrane glycoprotein.[10,28] Immunohistochemistry studies have demonstrated that PSMA is expressed in normal, benign, and malignant prostate epithelial cells but not in other normal adult tissues, with the possible exception of skeletal muscle,[8,11] which appears to show nonspecific staining (unpublished data). Overall, the data reported thus far continue to suggest that PSMA is a novel antigen with expression highly restricted to prostate tissues and that it may prove to be a useful biomarker for targeted diagnostic or therapeutic strategies or for gene therapy approaches. The MAb 7E11-C5.3 is the only reported antibody reactive to this glycoprotein, and it is therefore of interest to determine the epitope on PSMA that is reactive to this MAb. It is especially important to clarify the questions regarding the nature of the antigenic epitope with respect to the biochemical nature and location, as they could directly influence our understanding of the ability of CYT-356 to be useful in radioimmunoscintigraphy and immunotherapy. The present study examined the biochemical nature of PSMA and the epitope recognized by MAb 7E11-C5.3.

The basic physical and biochemical analysis of the PSMA epitope revealed that it is recognized in both a reduced and a denatured state based on the activity of the antigen in mercaptoethanol and SDS, respectively. Treatment of antigens with periodic acid has been used in the past to indicate the presence of carbohydrates in antigenic epitopes.[16] Therefore, the loss of activity after periodate treatment along with the sensitivity of PSMA to proteolytic digestion was initially interpreted to mean that the epitope consisted of a glycopeptide. In an attempt to determine the type of carbohydrate linkage in the epitope, the antigen was treated with sodium borohydride to cleave O-linked oligosaccharides; alternatively, LNCaP cells were grown in the presence of tunicamycin before antigen preparation to inhibit the addition of N-linked polysaccharides to the nascent peptide chain in the endoplasmic reticulum. Neither of these more specific treatments had any effect on MAb 7E11-C5.3 activity, contradicting the periodate results.

Lectin-binding experiments also indicated that carbohydrates were present in the epitope and that this carbohydrate component contained D-galactose in some form, because only lectins that were specific for either monomeric or polymeric galactose were able to block MAb 7E11-C5.3 binding. However, as with the basic biochemical analysis, we were unable to demonstrate specificity by either glycosidic digestion of PSMA or competitive binding experiments using specific monomeric or polymeric carbohydrates or aminosugars.

Because there was evidence to suggest that the antigenic epitope was intracellular, and considering the fact that there were only 19 amino acids in the intracellular domain,[10] peptides were synthesized to span this domain. The full-length 19-mer (N1-19) was active in both direct and competitive binding assays in a dose-dependent manner. Mapping of the peptide epitope by sequentially deleting residues from N1.19, one amino acid at a time, demonstrated that the minimal reactive peptide consisted of the first six N-terminal amino acids (MWNLLH). Deletion and substitution of the amino-terminal methionine or the carboxy-terminal histidine residue abrogated MAb 7E11-C5.3 binding, proving that these amino acids are essential for the integrity of the epitope. The affinity of MAb 7E11-C5.3 for N1.19 ($K_a$ $1.7 \times 10^9$ $M^{-1}$) was approximately 6.8-fold lower than the affinity for the native PSMA glycoprotein ($K_a$ $1.16 \times 10^{10}$ $M^{-1}$), whereas the affinity of MAb 7E11-C5.3 for N1.6 ($K_a$ $2.3 \times 10^8$ $M^{-1}$) was 7.4-fold lower than the affinity for N1.19. The direct peptide assays used in this study are a convenient means of assaying MAb

binding to peptides, which would otherwise be unable to adhere to the plastic of the assay plates, by covalently cross-linking the peptides with EDAC to BSA in the wells of the EIA plate. However, the peptides are covalently linked to BSA, which may significantly alter their conformation as compared to the native PSMA. It is not surprising or unprecedented, then, that the affinity constants for the peptides and the native antigen are slightly different.[29] This difference may be explained not only by the effects of cross-linking of the peptides to BSA, as discussed above, but also in differences between the linear synthetic peptides and the native PSMA molecule, which may have a significantly different conformation. In addition, modifications of the amino acids in the native protein, such as acetylation or other additions, might further stabilize the antigenic determinant of the native PSMA. Although the affinity of the N1.6 peptide may be lower than that of N1.19 and the native PSMA molecule for the same reasons as discussed above, the inhibition studies (Figure 5) seem to suggest that the smaller peptide does indeed have a lower affinity which most likely results from a destabilization of the antigenic determinant. Nevertheless, the epitope-mapping studies clearly demonstrate that the epitope recognized by MAb 7E11-C5.3 is the primary peptide chain of the intracellular domain of PSMA and that the minimal reactive peptide consists of the first six amino acids (MWNLLH) at the amino terminus.

It is important to note that several peptides are present in the extracellular domain of PSMA, which contains a motif similar to that of N1.6 (methionine at position 1 and histidine or a similar amino acid at position 6), but none of these peptides were active by direct binding or competitive binding assays. There was a possibility that these small peptides were not bound to the microtiter plates as efficiently as larger peptides. However, the competitive binding assay did not require that the peptides be bound and was an important control experiment to verify the negative results for these peptides observed in the direct binding assay. The size of the peptides may, however, cause a decrease in MAb 7E11-C5.3 affinity, as seen between N1.19 and N1.6, and experiments are underway to determine whether larger peptides from these regions are active.

The suggestion from the epitope-mapping data that the epitope consisted of only the core peptide and did not contain a carbohydrate moiety would explain the failure of the sodium borohydride, tunicamycin, and glycosidase treatments and of specific carbohydrates to block the MAb 7E11-C5.3 activity. However, the periodate oxidation and lectin competitive binding experiments were still contradicting if the MAb 7E11-C5.3 only binds to the peptide chain. Although periodate oxidation has been used successfully to identify carbohydrate epitopes, oxidation of amino acids also may occur.[30] The treatments used in this study (10 mmol/L periodate for 1 hour at 25°C) should have been mild enough to preclude damage to the polypeptide chain, while nonreducing sugars and pyranosidically linked hexoses within the oligosaccharide chains would have been oxidized.[16] Harsher conditions including longer incubations, higher temperatures, or higher periodate concentrations could have resulted in destruction of the peptide chain in addition to any carbohydrate. The possibility existed that the PSMA peptide epitope was particularly sensitive to periodate. This was especially possible because methionine residues are particularly susceptible to periodate oxidation.[30] In addition, the reactive peptides all began with the very N-terminal methionine of the protein, which may make it even more subject to oxidative attack because there is no protection on the amino-terminal end of this amino acid. Treatment of the reactive peptides N1.19 and N1.6 with periodate did, in fact, abrogate MAb 7E11-C5.3 binding. Oxidation of the peptide may result in cleavage or alteration of the amino acid residues. Therefore, to demonstrate the effects of either a loss or change in structure of the amino acids, peptides were synthesized with deletions or substitutions of either the methionine or the histidine residues, which also resulted in a loss of antibody binding. Clearly, both the methionine and the histidine residues were essential for activity of the peptides, and these experiments suggest that periodate oxidation was affecting the primary amino acid structure of PSMA.

Although it has been reported that PSMA can be detected in serum,[8,31] we and others have been unable to duplicate these results (unpublished data). The sensitivity of both the peptides and the native PSMA molecule to oxidation may explain the inability of MAb 7E11-C5.3 to detect the antigen in serum in our hands, as a result of either cleavage or blockage of the amino terminus by any number of serum factors. Experiments are underway to determine whether the native antigen, purified from LNCaP cells, or active PSMA peptides are stable in serum for extended periods. These data suggest that serum assays would be greatly improved if second-generation MAbs were created against more stable epitopes.

The ability of some lectins to block the MAb 7E11-C5.3 binding may have occurred as a result of nonspecific binding, because the native PSMA does contain a substantial amount of carbohydrate. The deglycosylated polypeptide has an Mr of 84,000, and the fully glycosylated molecule has an Mr of 100,000.[28] In addition, only lectins with a specificity for D-galactose were able to block MAb 7E11-C5.3 binding, although D-galactose in either monomeric or polymeric form was unable to compete for the MAb. The latter finding suggests that there may be a significant amount of galactose present on the native PSMA glycoprotein, but not in the antigenic epitope. If a number of D-galactose-containing oligosaccharides were close to but not directly in the antigenic epitope, the lectins could nonspecifically block MAb 7E11-C5.3 by steric hindrance and not by direct competition.

Although we have not mapped the entire PSMA molecule, the results of this study convincingly show that the epitope recognized by MAb 7E11-C5.3 is located in the intracellular domain of the PSMA transmembrane glycoprotein and consists solely of the polypeptide chain with a minimal reactive unit consisting of six amino acid residues (MWNLLH). An intracellular location of the MAb 7E11-C5.3 epitope introduces questions regarding the ability of CYT-356 to image living tumor masses. The immunoconjugated form of MAb 7E11-C5.3 (CYT-356) has been shown clearly to detect solid tumors in vivo[11–14] even though the epitope is intracellular. Possible explanations for this observation may be that CYT-356 images only necrotic tissues or those cells that have been lysed, either mechanically or via apoptosis, r that CYT-356 may be able to cross the plasma membrane to bind the an-

tigen. Alternatively, several peptides are present within the extracellular domain of PSMA: N470 (MYSLVH), N344 (MHIHST), N583 (MVFELA), and N664 (MNDQLM), which contain a motif similar to that of the N1.6 minimal reactive peptide. Although these peptides were not active by either a direct binding assay or a competitive binding assay, it is possible that CYT-356 may have a low affinity for any one of these peptides, which might result in a detectable signal upon immunoscintigraphy. Clearly, more studies are needed to determine definitively how CYT-356 is able to image solid tumors. Based on our findings that MAb 7E11-C5.3 binds to a peptide epitope localized to the intracellular domain, it would appear highly advantageous to produce second-generation antibodies to antigenic epitopes present in the extracellular domain of this transmembrane glycoprotein. Such antibodies could conceivably enhance the sensitivity of antibody-directed imaging and therapy applications, as well as provide excellent reagents for the development of in vitro diagnostic tests.

We would like to thank Judy Singleton for performing the tunicamycin experiments. We also thank Wendy Davis, Dr. Helena Axelrod, Dr. Vernon Alvarez, Dr. Dwight Lopes, Dr. Paul Kaladas, Dr. Maurice Rosenstraus, and Dr. Joseph V. Gulfo for helpful discussions and advice.

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The Prostate 30:232–242

# Location of Prostate-Specific Membrane Antigen in the LNCaP Prostate Carcinoma Cell Line

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**BACKGROUND.** Prostate-specific membrane antigen (PSMA) is a novel prostate biomarker overexpressed in poorly differentiated and metastatic prostate carcinomas and apparently upregulated following hormone-ablation therapy. PSMA appears to be a satisfactory target for antibody-directed imaging of prostate carcinomas despite the recent finding that the antigenic epitope recognized by monoclonal antibody (MAb) 7E11-C5 is found in the cytoplasmic domain of this transmembrane glycoprotein [Troyer et al.: Urol Oncol 1:29–37, 1995]. This finding prompted the present investigation to precisely define the cellular location of PSMA in the LNCaP prostate carcinoma cell line, the line used to generate MAb 7E11-C5.

**METHODS.** Subcellular fractionation, immunofluorescence and immunoperoxidase staining of live and fixed cells, and immunoelectron microscopy were used to determine the localization of PSMA in LNCaP cells.

**RESULTS.** PSMA was found to be localized at the inner face of the plasma membrane as well as being associated with mitochondria. Staining of LNCaP cells, treated by serum starvation followed by serum stimulation, showed no changes in the typical cytoplasmic staining pattern.

**CONCLUSIONS.** The data suggest that the PSMA target epitope for antibody-directed imaging with MAb 7E11-C5 only becomes accessible upon apoptosis or necrosis. This further suggests that antibodies directed at the extracellular domain may enhance the sensitivity of antibody-directed imaging and therapy of prostate carcinomas by recognizing surface epitopes of PSMA on living cancer cells. *Prostate 30:232–242, 1997.* © 1997 Wiley-Liss, Inc.

*KEY WORDS:* **tumor; biomarker; cancer; tumor antigen; PSMA**

## INTRODUCTION

Prostate cancer is the most commonly diagnosed adenocarcinoma and the second most common cause of cancer deaths in men in the United States [1]. The number of deaths attributed to prostate cancer is increasing at a rate of approximately 8% a year [1], which means that between 1995 and the year 2000, 2 million males will be diagnosed and of these approximately 300,000 will have died in just a 5-year time span. Therapy options for prostate cancer have not improved over the past decade and remain limited. Additionally, few model systems exist which allow for the study of novel therapeutic modalities. Therefore, in order to advance the treatment options for prostate cancer, novel strategies for diagnosis and therapy will be needed to improve the life expectancy of patients diagnosed with this disease.

The use of monoclonal antibody (MAb)-directed imaging and therapy has shown great promise for improving the survival of prostate cancer patients. However, improvements such as increased sensitivity and specificity must be realized before many of these antibody-directed techniques can be routinely utilized for clinical applications. These improvements

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are entirely dependent on an in-depth understanding of the MAb, the antigenic epitope recognized by the antibody, and the molecule which carries the epitope. With this knowledge in hand, appropriate modifications may be made to improve existing applications.

The prostate-specific membrane antigen (PSMA) is recognized by the MAb 7E11-C5.3 which was first described by Horoszewicz et al. in 1987 [2]. It appears to be overexpressed in poorly differentiated and metastatic prostate carcinomas [3] and antibody-radionucleotide conjugates have been successfully used to localize metastatic disease in vivo [4,5] and to treat human prostate tumors in nude mice [6]. These reports suggest PSMA may have promise as an important new diagnostic and therapeutic tool for prostate cancer. Like many clinical strategies, the sensitivity of MAb 7E11-C5.3 conjugates for use in the diagnosis or treatment of prostate cancer may depend on the accessibility of the 7E11-C5.3 epitope within a tumor mass. Previous reports have indicated that the MAb 7E11-C5.3 epitope on PSMA is located in the proposed intracellular domain of the PSMA molecule [7] which would seem to hinder the ability of MAb 7E11-C5.3 to bind to PSMA in intact cells. In the present study, the definitive immunolocalization of MAb 7E11-C5.3 in LNCaP cells has been determined by subcellular fractionation, and immunofluorescence and immunoelectron microscopy.

## MATERIALS AND METHODS

### Monoclonal and Polyclonal Antibodies

The MAb 7E11-C5.3, purified by protein-A affinity chromatography from murine ascites, was provided by Cytogen Corporation (Princeton, NJ). The MAb concentration was determined using a single radial immunodiffusion system (TAGO, Burlingame, CA). MAbs OKT-9 (Ortho Immunology Systems, Raritan, NJ), MU213 (BioGenex, San Ramon, CA), and proliferating cell nuclear antigen (PCNA; DAKO Corporation, Carpinteria, CA) were purchased and utilized according to the manufacturers' recommendations.

### Tissue Culture

LNCaP cells were obtained from the American Type Culture Collection (Rockville, MD) and were grown in RPMI 1640 medium supplemented with L-glutamine and 5% fetal calf serum (Gibco-BRL, Gaithersburg, MD) at 37°C and 5% carbon dioxide.

### Mechanical Subcellular Fractionation

The method employed here was modeled after several studies found in the literature [8–10]. Briefly, cultured LNCaP cells ($2 \times 10^8$) were harvested and lysed in a hypotonic medium (1 mM $NaHCO_3$), then dounce homogenized with 50 up and d wn strokes. It was imperative that the majority f the cells were lysed since the initial centrifugation steps would pellet out unbroken cells as well as nuclei. Therefore, the homogenization was monitored by trypan blue staining and the subsequent steps were not performed until >99% of the cells were lysed. The homogenate was centrifuged at 500*g* for 5 min at 4°C and the pellet resuspended in buffer containing 16% sucrose then underlayed with 20% sucrose and centrifuged in a swinging bucket rotor at 150,000*g* for 60 min at 4°C. The nuclei were pelleted to the bottom of the 20% sucrose while other membrane components remained at the interface of the two layers. The supernatant was removed by aspiration and the nuclear pellet resuspended in 16% sucrose and centrifuged again to remove any residual membranes since it has been shown to be difficult to obtain totally pure nuclei [10]. The supernatant from the 500*g* spin was centrifuged at 10,000*g* for 15 min at 4°C. The pellet from this step represented the heavy membrane fraction (HM) containing predominately the rough endoplasmic reticulum (ER), the Golgi apparatus, and mitochondria. The supernatant was centrifuged at 150,000*g* for 60 min at 4°C. The pellet from this step was the light membrane fraction (LM) containing smooth endoplasmic reticulum, plasma membrane, and any vesicular membranes while the supernatant represented the soluble cytoplasmic fraction (C).

The fractions obtained from this fractionation method were analyzed by Western blot and enzyme-linked immunosorbent assay (ELISA) for the presence of PSMA. Control antibodies, used as markers for mitochondria (MU213), plasma membrane (OKT-9), and nucleus (α PCNA), were utilized to verify that the partitioning was efficient.

### Living LNCaP Immunofluorescence Staining

LNCaP cells were grown to approximately 50% confluency on collagen-coated chambered slides (Nunc, Naperville, IL). The medium was removed by aspiration and the cells were washed twice with phosphate-buffered saline (PBS; 136 mM NaCl, 1.7 mM KCl, 8 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.4) warmed to 37°C. Blocking serum (10% goat serum in PBS) was added to the chambers and incubated at room temperature for 1 hr then removed by aspiration. The primary antibody (7E11-C5.3 at 20 μg/ml, OKT-9 at 15 μg/ml, PSA-5 at 1 μg/ml, or IgG1 isotype-matched control at 10 μg/ml) was added to the cells and incubated at room temperature in a humid chamber for 1 hr. The cells were washed twice with PBS followed by the

addition of secondary antibody (fluorescein isothiocyanate [FITC]-labeled goat F(ab') α mouse antibody with Evan's blue counter stain [Baxter Healthcare Corporation, West Sacramento, CA]) and incubated at room temperature for 1 hr. The cells were washed twice with PBS then visualized with a fluorescence microscope. Duplicate specimens were prepared for each antibody tested in the above experiment and all subsequent microscopic techniques used.

### Fixed LNCaP Cell Immunofluorescence Staining

LNCaP cells were grown to approximately 50% confluency on chambered slides as described above. The cells were fixed for 20 min in 10% buffered formalin and rinsed twice with PBS. The cells were permeabilized briefly in 0.1% Triton-X100 in PBS for 5 min, washed twice with PBS, then stained as described above using 7E11-C5.3, OKT-9, PSA-5, and the IgG isotype-matched negative control antibody.

### Immunoperoxidase Staining

The immunoperoxidase staining was carried out exactly as described above for the fixed immunofluorescence staining, except that the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) was used with a peroxidase-labeled horse α mouse antibody. The positive staining was visualized by the addition of a color substrate (diaminobenzidine [DAB]) for 10 min, then rinsed twice with PBS and counterstained with Mayer's hematoxylin for 5 min. Cytospin preparations were also made of LNCaP cells by first removing adherent cells from culture flasks using PBS-ethylenediaminetetraacetic acid (EDTA). Cells were washed twice in PBS and then resuspended in 10 ml PBS. Cytospin cups were loaded with 200 μl of cell suspension and spun at 400 rpm for 5 min. Slides were air dried and fixed for 20 min in 10% buffered formalin, followed by 5 min in 70% ethanol. The cytospin slides were then stained as described above for chambered slides.

### Immunoelectron Microscopy

To definitively determine the localization of the PSMA glycoprotein, a method for immunoelectron microscopy of PSMA was designed from previously reported studies in the literature [11–13]. LNCaP cells were grown to confluency on plastic coverslips. The coverslips were fixed in half-strength Karnovsky's fixative (2% paraformaldehyde and 1% glutaraldehyde in cacodylate buffer) for 30 min then osmicated in osmium tetroxide following standard conditions. The coverslips were embedded in LR-White and polymerized overnight at 4°C. Ultrathin sections were cut with a glass knife and mounted on nick grids. The grids w re stained using the hanging method by suspension on drops of blocking b (10% goat serum in filtered PBS) for 1 hr, then mary antibody (7E11.C5 or IgG control) for up t hr, rinsed by repeated hanging drop incubation wash buffer, then incubated with a secondary anti-mouse Ig antibody labeled with a 10 nm bead (Amersham Life Science, Arlington Hei IL). Following several rinses, the grids were coun stained by placement at the bottom of a drop of tered uranyl acetate for 15 min in the dark then rins three times by dipping 20 times for each wash in fre sterile water. After the final rinse, the grids we placed at the bottom of a drop of lead citrate an stained for 15 sec then rinsed three times as describe above. The grids were air dried on filter paper then analyzed on a JEOL transmission electron microscope.

### Serum Starvation Stimulation

LNCaP cells were grown to approximately 50% confluency on chambered slides as described above. The cells were washed three times with PBS warmed to 37°C, serum-free RPMI 1640 medium was added to each well, and the slides were incubated for an additional 48 hr at 37°C. Following serum starvation, the serum-free medium was removed by aspiration and replaced with RPMI 1640 medium supplemented with 5% calf serum and incubated at 37°C. Slides were removed at time points (0 min, 15 min, 30 min, 60 min, 2 hr, 4 hr, 6 hr, 8 hr, 10 hr, 24 hr, and 48 hr) following serum starvation and fixed for 20 min in 10% buffered formalin and stored in PBS at 4°C. After all of the time points were collected and fixed, the cells were permeabilized briefly in 0.1% Triton-X100 in PBS for 5 min, then washed twice with PBS. Immunofluorescence staining was carried out as described above using MAb 7E11-C5.3 and an IgG1 isotype-matched control antibody. All assays were run in triplicate.

### Mitochondrial Purification

Mitochondria were purified from LNCaP cells following a previously reported procedure [14]. Six 162 $cm^2$ tissue culture flasks were seeded with LNCaP cells and grown to confluency. The cells were harvested then pelleted by centrifugation. The cell pellet was resuspended in 6 ml 1 × mitochondrial isolation buffer (MIB; 0.25 M sucrose, 40 mM Tris, pH 7.0, 0.1 mM EDTA) then dounce homogenized on ice with 40 up and down strokes. The homogenized material was centrifuged in a Beckman JA20 rotor at 2,000 *g* for 30

min at 4°C, and the supernatant was collected and centrifuged at 8,500 *g* for 35 min at 4°C. The supernatant from this step was discarded, and the pellet resuspended in 6 ml 1 × MIB and centrifug d at 8,500 *g* for 35 min. The supernatant fr m this step was discarded and the pellet resuspended in 6 ml 1 × MIB and dounce homogenized. This suspension represented the crude mitochondrial fraction. The crude mitochondrial fraction was applied to a two-step sucrose gradient composed of 10 ml of 25% sucrose in TE buffer (10 mM Tris, 0.1 mM EDTA) and 13 ml of 42.5% sucrose in TE buffer and centrifuged at 26,000 *g* for 75 min at 4°C. The percentage of sucrose in each solution was confirmed by a refractive index of 1.3775 and 1.4035, respectively. The mitochondria were collected at the interface of the two sucrose layers and diluted in two volumes TE buffer and applied to a second sucrose gradient purification. The mitochondria were again collected at the interface of the two sucrose layers, diluted in 2 volumes TE buffer then centrifuged at 22,000 *g* for 20 min at 4°C. The pellet from this step, representing the purified mitochondria, was resuspended in 2 volumes PBS and stored at −20°C until needed.

## RESULTS

### CAP Cellular Subfractionation

Following subcellular subfractionation procedures carried out on cultured LNCaP cells, the PSMA glycoprotein was detected by Western blot analysis in both the HM and the LM fractions at approximately 100 kDa (Fig. 1). The HM fraction also contained a higher molecular weight band which was barely detectable in the LM fraction, indicating that two pools of this molecule exist in LNCaP cells. A small amount of reactivity was also seen in the nuclear fraction but may be due to contamination. A panel of control antibodies, including antibodies against the PCNA, HSP60 (MU213), the transferrin receptor (OKT-9), and prostate-specific antigen (PSA), was utilized to monitor the efficiency of the fractionation (data not shown).

### Immunofluorescence Microscopy of PSMA in LNCaP Cells

LNCaP cells grown on tissue culture slides were stained without fixation or permeabilization prior to immunofluorescence staining with MAb 7E11-C5.3 or a control antibody (OKT-9) specific for the extracellular domain of the transferrin receptor. The transferrin receptor antibody was able to bind its epitope on the extracellular face of the LNCaP cells, resulting in a ring of staining around the cells (Fig. 2A) as expected

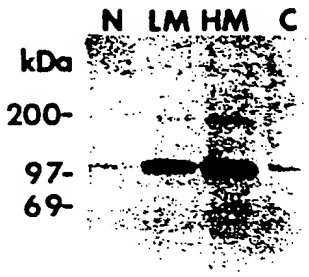


**Fig. 1.** Western blot analysis of cellular fractions. The majority of PSMA was found in the light membrane (LM) and heavy membrane (HM) fractions with very little in the nuclear (N) and cytoplasmic (C) fractions.

for this extracellularly expressed epitope. On the other hand, MAb 7E11-C5.3 was unable to bind to its epitope (Fig. 2B), indicating its epitope was intracellular.

To more definitively determine the localization of PSMA within the LNCaP cell line, cells grown on tissue culture slides were fixed with buffered formalin and permeabilized briefly in a weak detergent solution prior to staining. The isotype-matched control antibody did not stain the LNCaP cells (Fig. 2C) and all that was visible was the red counterstaining of the Evan's blue. Staining with PSA-5 showed a remarkable vesicular staining of the LNCaP cells (Fig. 2D), suggesting a localization in secretory vesicles. The transferrin receptor appeared to be predominantly localized to the plasma membrane (Fig. 2E), as demonstrated by the circumscription of the cell by the yellow fluorescence. The staining for PSMA with MAb 7E11-C5.3, however, appeared to be cytoplasmic and not localized at the plasma membrane or the nucleus (Fig. 2F). There was no circumscription of the cells as seen with the transferrin receptor and no vesicular staining similar to PSA.

A similar set of experiments were carried out using immunoperoxidase staining methods and visible light microscopy with identical results. The staining pattern for PSMA was observed to be cytoplasmic (Fig. 3A,B) while the transferrin receptor was restricted to the plasma membrane (Fig. 3C).

### Immunoelectron Microscopy of PSMA in LNCaP Cells

A series of immunoelectron microscopy studies were then p rformed to d finitively determine the

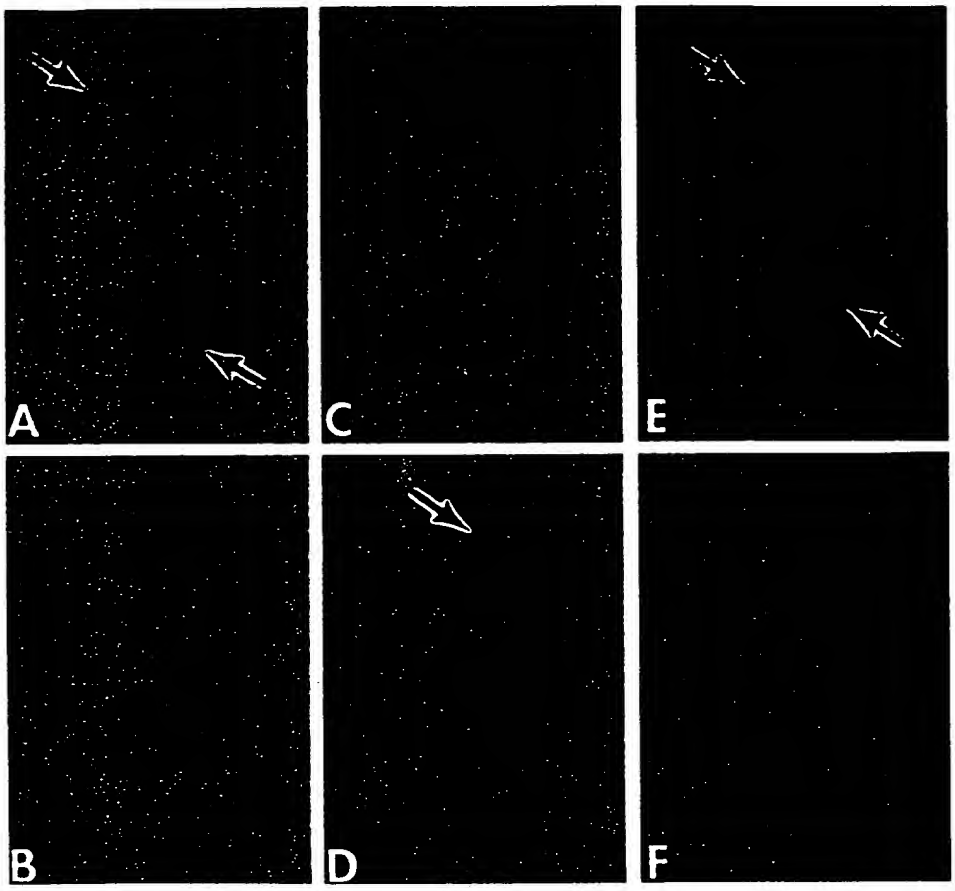


**Fig. 2.** Live and fixed cell immunofluorescent analysis of LNCaP cells. For the live cell assay, LNCaP cells were grown on tissue culture slides, washed, and reacted with MAb without fixation. **A:** Positive staining (yellow-green ring around cells indicated by arrows) of the extracellular domain of the transferrin receptor with MAb OKT-9. **B:** MAb 7E11-C5 did not react with LNCaP cells under the same conditions. Only the Evan's blue counterstain (red fluorescence) is observed. Fixed LNCaP cells were reacted with either the IgG1 isotype-matched control (**C**), MAb PSA-5 (**D**), MAb OKT-9 (**E**), or MAb 7E11-C5 (**F**). The isotype-matched antibody control was negative while PSA-5 gave a very vesicular staining pattern (arrow). OKT-9 staining was similar to that observed in the live cell assay in that the staining was confined to the plasma membrane (arrows), whereas LNCaP cells stained with MAb 7E11-C5 appeared to be predominantly cytoplasmic and not localized to the nucleus or exclusively to the plasma membrane.

intracellular localization of PSMA within the cytoplasm of LNCaP cells. The first set of experiments utilized a chromagen (DAB) appearing as electron-dense particles to visualize PSMA staining. Figure 4 shows the positive staining of MAb 7E11-C5.3 at the cytoplasmic face of the cell membrane (CM) and microvilli (MV) (Fig. 4A,B) and within several mitochondria (Fig. 4G).

To more precisely demonstrate the localization of PSMA, a secondary MAb directly labeled with a 10 nm gold bead was used in place of chromagen staining. Immunogold staining with MAb 7E11-C5.3 also resulted in positive staining with a concentration of gold beads at the cytoplasmic face of the CM and near a gap junction (GJ) between two LNCaP cells (Fig. 4E) and concentrated in and around mitochondria (Fig. 4H). When LNCaP cells were reacted with MAb OKT-9, immunogold staining was localized to the outer face of the plasma membrane (Fig. 4D). Specific staining of the plasma membrane or mitochondria was not observed when an isotope-matched MAb was used (Fig. 4C,F).

### PSMA Identified in Mitochondria

Since two separate experiments suggested that PSMA was, in part, localized to the mitochondria of LNCaP cells, mitochondria were purified from whole cell lysates and analyzed for the presence of PSMA. The 100 kDa PSMA glycoprotein was present in the

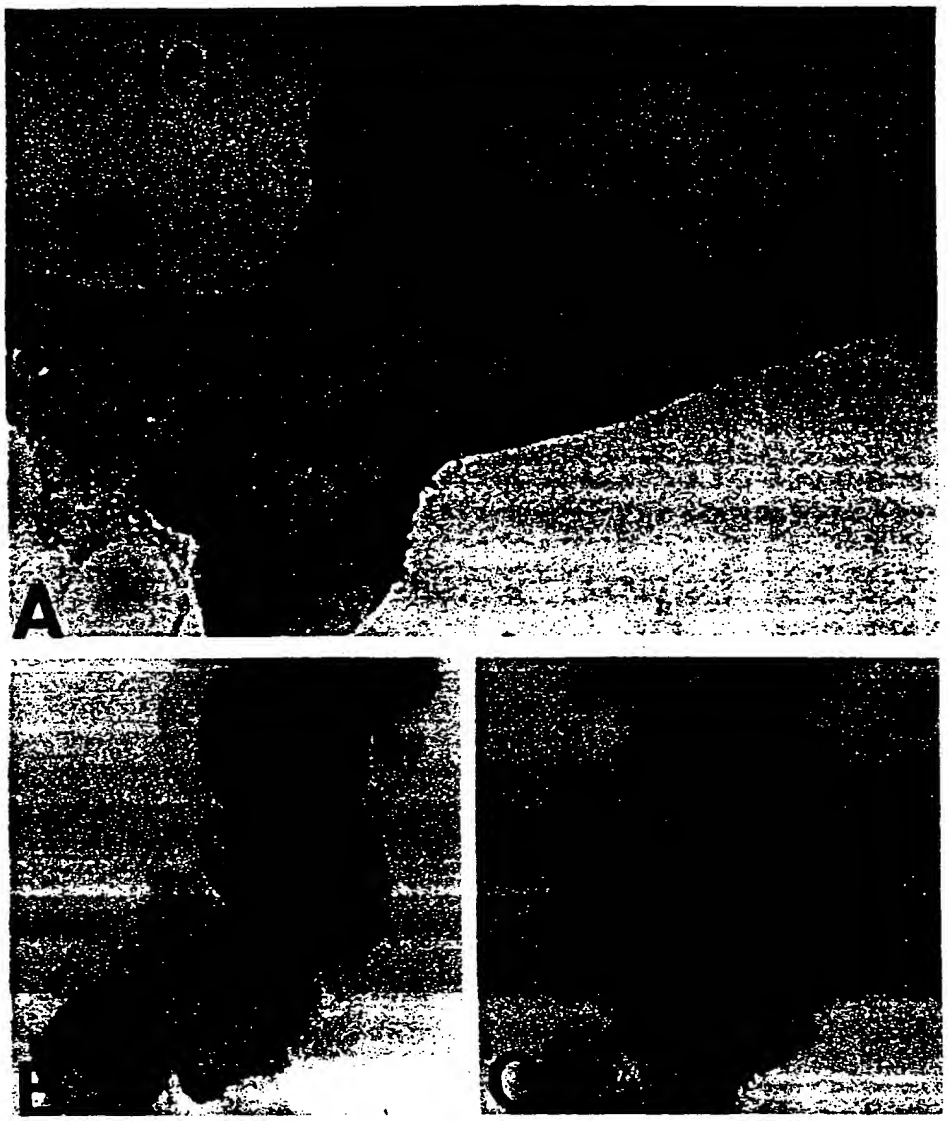


**Fig. 3.** Immunoperoxidase staining of LNCaP cells. **A:** Cultured LNCaP cells, fixed, and reacted with MAb 7E11-C5 show intense cytoplasmic staining. **B:** Cytospin preparation of fixed LNCaP cells reacted with MAb 7E11-C5 also shows intense cytoplasmic staining. **C:** Cytospin preparation of fixed LNCaP cells reacted with the anti-transferrin receptor MAb OKT-9. In contrast to the cytoplasmic staining with MAb 7E11-C5, MAb OKT-9 results in the ring membrane staining pattern typical for the location of an extracellular membrane epitope.

LNCaP lysate (Fig. 5, lane 1), crude mitochondrial preparation (Fig. 5, lane 2), and purified mitochondria following several sucrose gradient centrifugation steps (Fig. 5, lane 3), adding further evidence that PSMA is associated with the mitochondria of LNCaP cells.

Further evidence for PSMA association with mitochondria was shown by copurification of a mitochondrial protein with PSMA. A third major band of an approximate molecular weight of 40 kDa was copurified with PSMA on a MAb 7E11-C5.3 affinity column when low detergent wash buffers were used on the column (Fig. 6, lane 1), but was not immunoreactive (lane 2). This band was blotted to nitrocellulose and subjected to amino acid sequence analysis. The amino acid sequence of this band was completely identical to the precursor form of the mitochondrial isoenzyme of aspartate aminotransferase (m-AST), which is also known as S glutamic oxalacetic transaminase (SGOT) (data not shown).

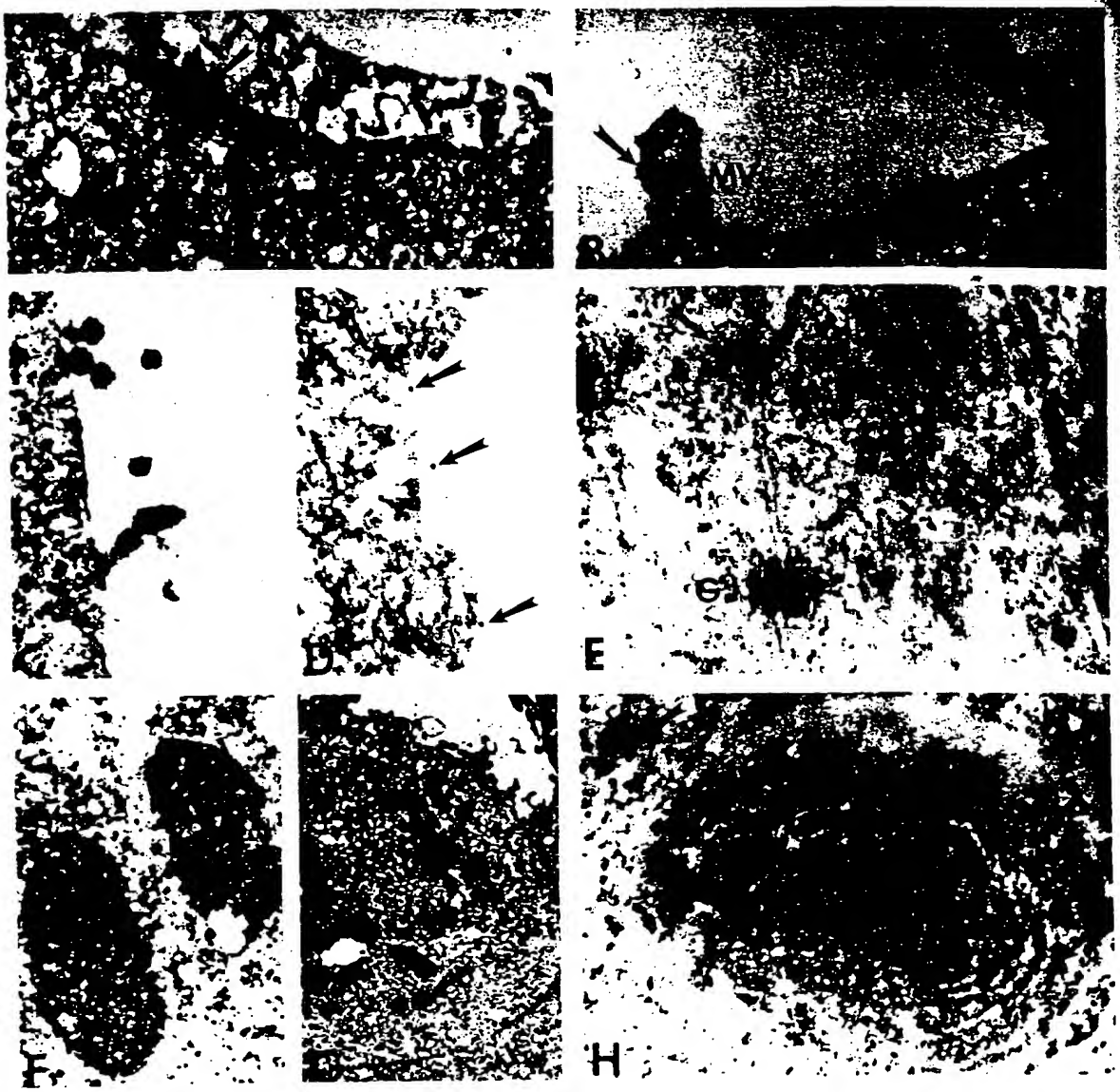


**Fig. 4.** Immunoelectron microscopy of LNCaP cells. Location of the PSMA-7E11-C5 complex was detected using either a peroxidase (DAB; Panels A, B, F, and G) or colloidal gold (Panels C–F) procedure as described in Materials and Methods. The PSMA-antibody complex was occasionally found at the cytoplasmic side of the cell membrane (CM) (**A**, arrow), and microvilli (MV; Panel **B**, arrow). No DAB or gold beads were found to be localized to the cell membrane in cells reacted with an isotype control MAb (negative control) (**C**). **D**: Location of gold beads at the outer face of the plasma membrane (arrows) of cells reacted with MAb OKT-9 (positive membrane control). The most common location of the PSMA-7E11-C5 complex was at the inner face of the plasma membrane (**E**) and in mitochondria (**G, H**). Panel E shows a cluster of gold beads under the cell membrane (CM) (arrow) near a gap junction (GJ). Panels F, G are examples of mitochondria reacted with either the isotype control MAb (**F**), or with MAb 7E11-C5 (G, H). Immune complexes were not detected with the control antibody (F), whereas PSMA was localized within and around (arrows, G and H) mitochondria with MAb 7E11-C5. Magnifications: ×13,000 (G); ×28,000 (C, F); ×81,000 (B, D); ×97,000 (A, E, H).

### Effects of Serum Starvation and Stimulation on PSMA Localization

LNCaP cells examined after serum starvation followed by serum stimulation showed the typical cytoplasmic immunofluorescent staining pattern of PSMA observed under normal growth conditions, suggesting that the localization and expression of PSMA was not dependent on extracellular signals.

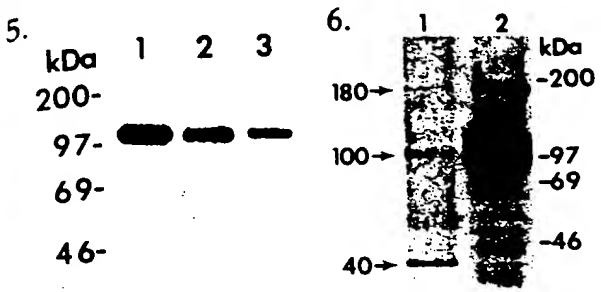


Fig. 5. Western blot analysis of crude and purified mitochondria. The mitochondria were purified by a series of centrifugation steps and sucrose gradient centrifugation. The LNCaP cell lysate contained a significant amount of the 100 kDa PSMA glycoprotein (lane 1) as did the crude mitochondrial preparation (lane 2) and the pure mitochondria following two sucrose gradient centrifugation purifications (lane 3), indicating that PSMA is found in the mitochondrial fraction of LNCaP cells.

Fig. 6. Identification of the mitochondrial subunit SGOT-AST copurified with PSMA. Affinity-purified PSMA was separated by gel electrophoresis and the gel either stained with coomassie blue (lane 1) or immunoblotted with MAb 7E11-C5 (lane 2). Both the 100 and 180 kDa PSMA bands were detected by staining and immunoblotting, with the 100 kDa band being highly reactive (lane 2). Note the prominent 40 kDa SGOT-AST band, which copurified with PSMA (lane 1), was not immunoreactive with MAb 7E11-C5 (lane 2).

## DISCUSSION

PSMA is a transmembrane glycoprotein found to be predominately expressed by normal, benign, and malignant prostate epithelial cells and overexpressed in high-grade and metastatic prostate carcinomas [3]. The function of PSMA remains unknown. Recently, we reported that the antigenic epitope recognized by MAb 7E11-C5 was located on the intracellular or cytoplasmic domain of PSMA [7]. Interestingly, MAb 7E11-C5 conjugated to $^{111}$In has been successfully used to image metastatic prostate carcinomas in vivo [4,5]. It is currently believed that for most antibodies to be effective for in vivo imaging of tumors, they must bind to an extracellular or surface membrane antigen. Since it appeared from our previous studies [3,7] that MAb 7E11-C5 bound to a cytoplasmic antigen, how does MAb 7E11-C5 image occult prostate carcinomas if it cannot penetrate the plasma membrane of the intact cancer cell? The present investigation was performed to address this issue and to provide definitive information about the cellular localization of PSMA. The LNCaP prostate cell line was selected for this study because it was the cell line used to generate MAb 7E11-C5.

Live cell immunofluorescence staining of LNCaP cells showed that MAb 7E11-C5 was unable to bind its target epitope, in contrast to a MAb to the transferrin receptor which expectedly bound to its epitope at the extracellular face of the plasma membrane. When fixed and permeabiliz d LNCaP were stained with MAb 7E11-C5, a diffuse cytoplasmic staining pattern was observed. This cytoplasmic staining pattern was observed by both immunofluorescent and immunoperoxidase methods, suggesting that the cytoplasmic staining was not due to an artifact of the staining method. It may, nevertheless, be argued that the weak detergent permeabilization of the cells prior to staining caused the cell membrane to dissipate, resulting in the cytoplasmic staining pattern. However, cells treated in the same manner and stained with MAb OKT-9 to the transferrin receptor showed the typical membrane staining [15] around the circumference of the cells, indicating that detergent treatment was not sufficient to cause diffusion or loss of the membrane epitope. These findings strongly supported a cytoplasmic location of the PSMA epitope recognized by MAb 7E11-C5.

We next attempted to pinpoint the intracellular location of PSMA by performing cellular immunoelectron and subfractionation experiments. Immunoelectron microscopy of LNCaP cells showed that PSMA was in fact found at two locations inside the cell. The PSMA epitope recognized by MAb 7E11-C5.3 was repeatedly found localized at the cytoplasmic face of the plasma membrane, strongly supporting the conclusion of an intracellular epitope. The only distinct positive staining in addition to the plasma membrane was found within and surrounding the outer mitochondrial membrane. A localization solely at the cytoplasmic face of the cell membrane would result in a ringed staining identical to that of an extracellular epitope. However, the additional localization to mitochondria would explain the diffuse cytoplasmic appearance of the staining at the light microscopy level. Positive staining for PSMA at both the cell membrane and mitochondria was observed utilizing both indirect chromagen staining and direct staining with a gold-labeled secondary antibody. These identical results using multiple immunoelectron microscopy techniques, as well as the negative staining by an isotype-matched control antibody, suggest the staining was specific.

Although the controls used for the immunolocalization experiments strongly suggested that our conclusions for the intracellular location of PSMA wer valid, we felt that further experiments were needed to prove that PSMA was found in or associated with the mitochondria. When purified mitochondria from LNCaP cells were tested by Western blot analysis, a high concentration of PSMA was observed. Although

the purity of the purified mitochondrial preparation was not examined by electron microscopy, the purification procedure used routinely yields a highly purified preparation [14]. Furthermore, it seems unlikely that a small amount of contamination with other cellular membranes would have given such a strong signal upon immunoblotting with MAb 7E11-C5.3.

The cellular subfractionation of LNCaP cells also clearly demonstrated two pools of PSMA; one pool in the LM fraction most likely consisting largely of plasma membrane components indicated by the high concentration of PSMA measured by ELISA and Western blot analyses; and the other in the HM fraction containing mostly intracellular organelles. Only very small amounts of PSMA were found in either the cytoplasmic or nuclear fractions. The HM fraction may also contain a significant number of plasma membrane proteins that happen to be contained in the rough ER as they transit from the ER to the membrane. The concentration of these plasma membrane components in the ER may be enhanced if there is a defect in trafficking of molecules through this pathway [16]. Alternatively, PSMA may be found in the mitochondria of LNCaP cells since the mitochondria make up a significant portion of the HM fraction as judged by the detection of significant concentrations of the mitochondrial-specific Hsp60 protein.

Collectively, the data from the different experiments described in this report strongly suggest the PSMA glycoprotein is localized at the plasma membrane with the epitope facing the cytoplasm and that it is also present within mitochondria. While it was quite unexpected to find a prostate-specific glycoprotein localized to an organelle considered to be rather generic in its constitution, there is precedent for the localization of plasma membrane glycoproteins to the mitochondria. For example, the Her2/neu oncogene product has also been shown to be localized at the plasma membrane and mitochondria [17]. Interestingly, like PSMA, a large percentage of Her2/neu staining in prostate and breast carcinoma cells appears to be cytoplasmic in nature [18,19] and not localized solely at the plasma membrane. This would seem to suggest there is a significant amount of communication between the extracellular environment and intracellular organelles. It is likely that a monitoring mechanism exists which may modify the metabolic activity of the organelle and that a significant number of plasma membrane receptors, including Her2/neu and PSMA, may be involved in this process. While such a mechanism may not have been proposed 10 years ago, a similar communication has been described among the mitochondria, peroxisomes, and the nucleus [20] and during starvation, myocardial cells appear to have an adaptive interaction between the intracellular and extracellular environments [21]. Additionally, many studies have been carried out in recent years on the organization of the extracellular matrix, concluding that cells seem to be able to sense the macromolecular composition of the extracellular matrix and to modify their production of matrix components accordingly [22]. These studies indicate that a considerable amount of communication occurs between the different cellular components. Therefore, the suggestion that PSMA may be involved in subcellular communication is not without precedent.

Serum starvation or stimulation of LNCaP cells failed to alter the staining pattern of PSMA. It is possible that a signal at the cell membrane results in a sequestration of PSMA at the membrane and if the ligand for PSMA is not present in the tissue culture media, PSMA would remain spread throughout the cell. In fact, a detailed examination of MAb 7E11-C5.3 staining of prostate tissue showed some ductal epithelial cells to exhibit staining confined to the plasma membrane with no cytoplasmic staining [3]. This staining pattern may reflect the presence or absence of a possible PSMA ligand which has not been duplicated in the in vitro studies.

Although the results of this study do not provide a definitive answer to why $^{111}$In-conjugated MAb 7E11-C5 (CYT-356) can image prostate carcinomas in vivo, it is doubtful CYT-356 is able to cross the plasma membrane since 7E11-C5.3 was not able to do so in vitro in living LNCaP cells. Due to the propensity of prostate cells to enter apoptosis [23], the most probable answer is that a significant number of cells within a tumor may be lysed following cell death, thereby uncovering the antigenic epitope. The number of apoptotic cells within a tumor may be substantially increased if the patient is on hormone ablation therapy since withdrawal of androgen hormones induces apoptosis [24], which may lead to an increase in the number of epitopes available to be recognized by CYT-356. Preliminary evidence suggests that hormone ablation therapy can upregulate the expression of PSMA [25] [Wright et al., unpublished observations], thereby supporting this possibility. Alternatively, as a tumor grows, a significant portion of the tumor volume may become necrotic [26], again allowing for access to the intracellular epitope. Both of these mechanisms would result in CYT-356 "seeing" only a small percentage of the cells within a given tumor mass resulting in a significantly low sensitivity. As a result, specific second-generation antibodies generated against the extracellular domain of PSMA may dramatically increase the sensitivity of imaging assays by increasing the number of cells which can be

recognized by the antibody. This increased sensitivity may result in the detection of smaller metastatic foci containing a large percentage of intact cells which would not be discovered by MAb 7E11-C5. It must be stated in any discussion of PSMA second-generation antibodies, however, that the MAb 7E11-C5.3 may be the most selective antibody for prostate tissue. It is highly likely that many of the MAbs generated against the extracellular domain of PSMA may be crossreactive with other cellular antigens. Therefore, it may not be possible to greatly improve the sensitivity of PSMA imaging over that which is obtained using 7E11-C5.3

In summary, the results of this study strongly support the intracellular location of the PSMA epitope recognized by MAb 7E11-C5, not only to the cytoplasmic face of the plasma membrane but in association with mitochondria. This latter observation may offer a clue to the function of the PSMA glycoprotein. Further, since MAb 7E11-C5 cannot enter living LNCaP cells to reach its target epitope, it apparently binds to PSMA when it is released from cells undergoing apoptosis or necrosis. These observations would suggest that generating MAbs to extracellular epitopes may enhance the use of antibody-directed imaging and therapy of prostate carcinomas, and possibly for development of other potential clinical applications of this novel prostate cancer biomarker.

## ACKNOWLEDGMENTS

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*Advances in Brief*

# Monoclonal Antibodies to the Extracellular Domain of Prostate-specific Membrane Antigen Also React with Tumor Vascular Endothelium[1]

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## Abstract

**Prostate-specific membrane antigen (PSMA), initially defined by monoclonal antibody (mAb) 7E11, is a now well-characterized type 2 integral membrane glycoprotein expressed in a highly restricted manner by prostate epithelial cells. 7E11 has been shown to bind an intracellular epitope of PSMA that, in viable cells, is not available for binding. Herein, we report the initial characterization of the first four reported IgG mAbs that bind the external domain of PSMA. Competitive binding studies indicate these antibodies define two distinct, noncompeting epitopes on the extracellular domain of PSMA. In contrast to 7E11, these mAbs bind to viable LNCaP cells *in vitro*. In addition, they show strong immunohistochemical reactivity to tissue sections of prostate epithelia, including prostate cancer. These mAbs were also strongly reactive with vascular endothelium within a wide variety of carcinomas (including lung, colon, breast, and others) but not with normal vascular endothelium. These antibodies should prove useful for *in vivo* targeting to prostate cancer, as well as to the vascular compartment of a wide variety of carcinomas.**

## Introduction

PSMA[3] is a highly restricted prostate epithelial cell membrane glycoprotein of approximately 100 kDa (1, 2). The PSMA gene has been cloned, sequenced (2), and mapped to chromosome 11q14 (3). In contrast to other highly restricted prostate-related antigens such as PSA, prostatic acid phosphatase, and PSP, which are secretory proteins, PSMA is an integral membrane protein. Among the reasons for significant interest in PSMA is that it is ideal for *in vivo* prostate-specific targeting strategies. In addition to its prostate specificity (1, 2, 4, 5), PSMA is expressed by a very high proportion of PCas (6); this expression is further increased in higher-grade cancers, in metastatic disease (6), and in hormone-refractory PCa (5–7).

The initial validation of PSMA as an *in vivo* target has been borne out by imaging trials with mAb 7E11/CYT-356 (8–11). However, epitope mapping indicates that 7E11/CYT-356 targets an intracellular epitope (12, 13). In viable cells, this binding site is not accessible to an antibody (1, 13). Successful imaging with 7E11/CYT-356 probably relates to the targeting of dead/dying cells within tumor sites (6, 12, 13). It has been noted (2, 12–14) that a mAb to the extracellular domain would provide benefits, including improved *in vivo* localization and enhanced imaging and therapy. In this study, we report the development of four IgG mAbs to the external domain of PSMA. These mAbs also have been found reactive to vascular endothelium within a wide range of carcinomas but not with normal endothelial cells.

## Materials and Methods

**Generation and Production of mAb.** BALB/c mice were immunized three times with LNCaP cells or a primary culture of PCa epithelial cells. Spleen cells were fused with X63.Ag.653 mouse myeloma cells using standard hybridoma technique. Clones that were reactive against LNCaP but unreactive against tissue sections of normal kidney (with the exception of some proximal tubule reactivity) and colon were subcloned. Murine ascites fluid was produced, and mAbs were purified using protein G (Pharmacia LKB Biotechnology, Piscataway, NJ). Purified mAb 7E11 was a generous gift from Dr. Gerald P. Murphy (Pacific Northwest Research Foundation, Seattle, WA).

**Immunohistochemical Staining.** Normal and cancer tissues were precooled in liquid nitrogen, snap-frozen in OCT compound (Miles Inc., Elkhart, IN) on dry ice, and stored at −80°C. Cryostat tissue sections (5 μm) were fixed in cold acetone (4°C) for 10 min. mAbs (5 μg/ml or hybridoma supernatants) were incubated for 1 h at RT. Antibody binding was detected using rabbit antimouse immunoglobulin-peroxidase (DAKO Corp., Carpinteria, CA) as a secondary antibody and diaminobenzidine (Sigma Chemical Co., St. Louis, MO) as chromogen. Isotype-matched irrelevant antibody was used as a negative control.

**Cross Immunoprecipitation.** LNCaP cells were lysed in lysis buffer [20 mM Tris/HCl, pH 8; 1 mM EDTA; 1 mM phenylmethylsulfonyl fluoride; and 1% (v/v) Triton X-100]. The resulting lysate was precleared by incubation with protein G beads overnight at 4°C, then incubated with mAb for 2 h. Protein G beads were added for 90 min prior to further washing. The beads were resuspended and boiled for 5 min in 1× Laemmli sample buffer containing 2-mercaptoethanol at 5% final concentration. The samples were centrifuged, and supernatant was recovered and placed on a 12% SDS-PAGE gel. After electrophoresis, the separated proteins were transferred to a nitrocellulose membrane. The membranes were blocked with 5% dry milk/TBST (Tris-buffered saline-Tween 20), incubated with primary mAbs × 60 min and followed by sheep antimouse immunoglobulin-peroxidase (Amersham Corp., Cleveland, OH). After washing, the membranes were developed using the enhanced chemiluminescence method (Amersham). Either 0.15 μg/ml J591 or 0.5 μg/ml 7E11 was used as a probe to detect the protein that was immunoprecipitated by 7E11, J591, J533, J415, and E99, respectively. Isotype-matched irrelevant antibody (156; reactive to PSP) was used as a negative control.

**IF Assay.** LNCaP cells were grown on glass coverslips. IF assays were performed using either viable or fixed cells, the latter being either permeabilized or nonpermeabilized. For fixation, cells were treated with 2% paraformaldehyde-PBS (PBS, pH 7.4) for 30 min at RT, which does not permeabilize the cell membrane, washed with 1% BSA-PBS, quenched for 10 min in 50 mm $NH_4Cl$ in PBS, and rinsed with 1% BSA-PBS. Where cell membrane perme-

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[3] The abbreviations used are: PSMA, prostate-specific membrane antigen; PSA, prostate-specific antigen; IEM, immunoelectron microscopy; IF, immunofluorescence; mAb, monoclonal antibody; PCa, prostate cancer; PSP, prostate secretory protein; RT, room temperature.

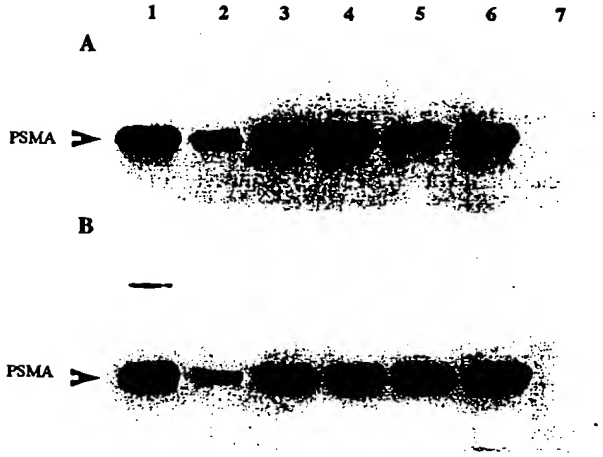


Fig. 1. Cross immunoprecipitation shows mAbs J591, J415, J533, E99, and 7E11 recognize the same antigen. *Lane 1*, crude LNCaP lysate. In *Lanes 2-7*, LNCaP lysate was immunoprecipitated with 5 μg/ml mAb 7E11 (*Lane 2*); 5 μg/ml J591 (*Lane 3*); 10 μg/ml J533 (*Lane 4*); 10 μg/ml J415 (*Lane 5*); 10 μg/ml E99 (*Lane 6*); and 10 μg/ml I56 (to PSP; *Lane 7*). Immunoprecipitates were immunoblotted with 0.15 μg/ml mAb J591, (*A*) or 0.5 μg/ml 7E11 (*B*).

abilization was desired, 0.075% saponin (Sigma) in 1% BSA-PBS was added for 15 min at RT.

Primary mAb at 4 μg/ml in BSA-PBS (plus saponin in cases of permeabilization) was incubated for 60 min at 4°C in the case of viable cells or at RT for fixed cells. After primary mAb incubation, viable cells were fixed in cold methanol for 20 min. FITC-goat antimouse secondary antibody (1:100 in BSA:PBS ± saponin; Jackson ImmunoResearch, West Grove, PA) was incubated for 30 min and washed extensively in 1% BSA-PBS. Slides were mounted in Vectashield (Vector Laboratories, Inc., Burlingame, CA).

**IEM Microscopy.** The IEM procedure was similar to the nonpermeabilized IF assay above. LNCaP cells were grown in 35-mm culture dishes and incubated with 10 μg/ml J591 or 10 μg/ml 7E11 for 45 min at 4°C, fixed with 2% paraformaldehyde, washed, and quenched as above. After 1% BSA washes, cells were incubated with 15-nm gold-conjugated goat antimouse IgG (Amersham) for 1 h. After extensive washing, cells were fixed in 2.5% glutaraldehyde for 15 min, gently scraped, pelleted, and processed for IEM as described previously (15). Electron micrographs were taken with a Joel 100CX electron microscope.

**Competitive Binding Assay.** Biotinylated mAbs were prepared by incubating 1 mg/ml mAb with 0.1 ml of biotinamidocaproate *N*-hydroxysuccinamide ester (1 mg/ml; Sigma) in DMSO for 4 h at RT. Unbound biotin was removed by dialysis against PBS. 7E11 (10 μg/ml) was coated onto Terasaki plates. Plates were washed with 1%BSA-PBS-0.1% Tween 20. LNCaP membrane preparations were added to wells for 1 h at RT. After washing, serial dilutions of unlabeled (competing) antibody were added to duplicate wells for 1 h. Biotinylated antibody was added to each well and incubated for an additional 1 h followed by avidin-alkaline phosphatase (Sigma). After wash-

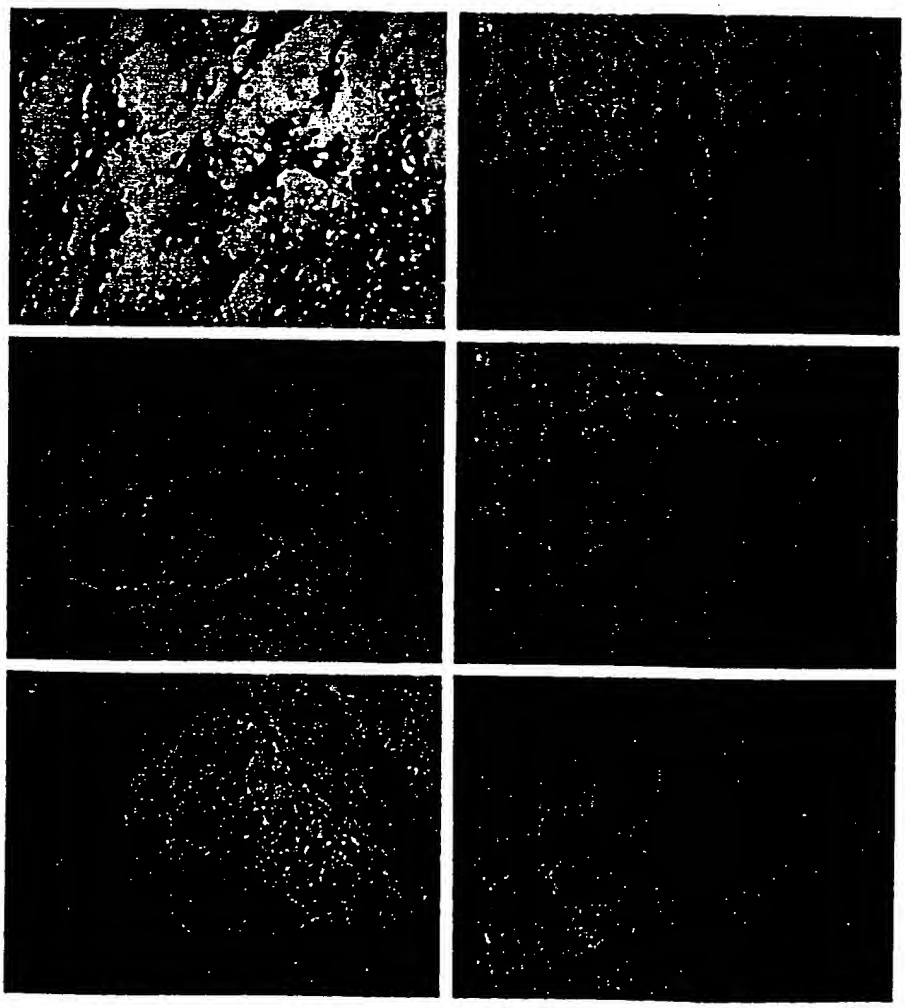

Fig. 2. Immunohistochemical reactivity of mAb J591 to neovasculature of renal (*A*), urothelial (*B*), colon (*C*), lung (squamous; *D*), breast carcinomas (*E*), and metastatic adenocarcinoma to liver. (*F*). ×250.

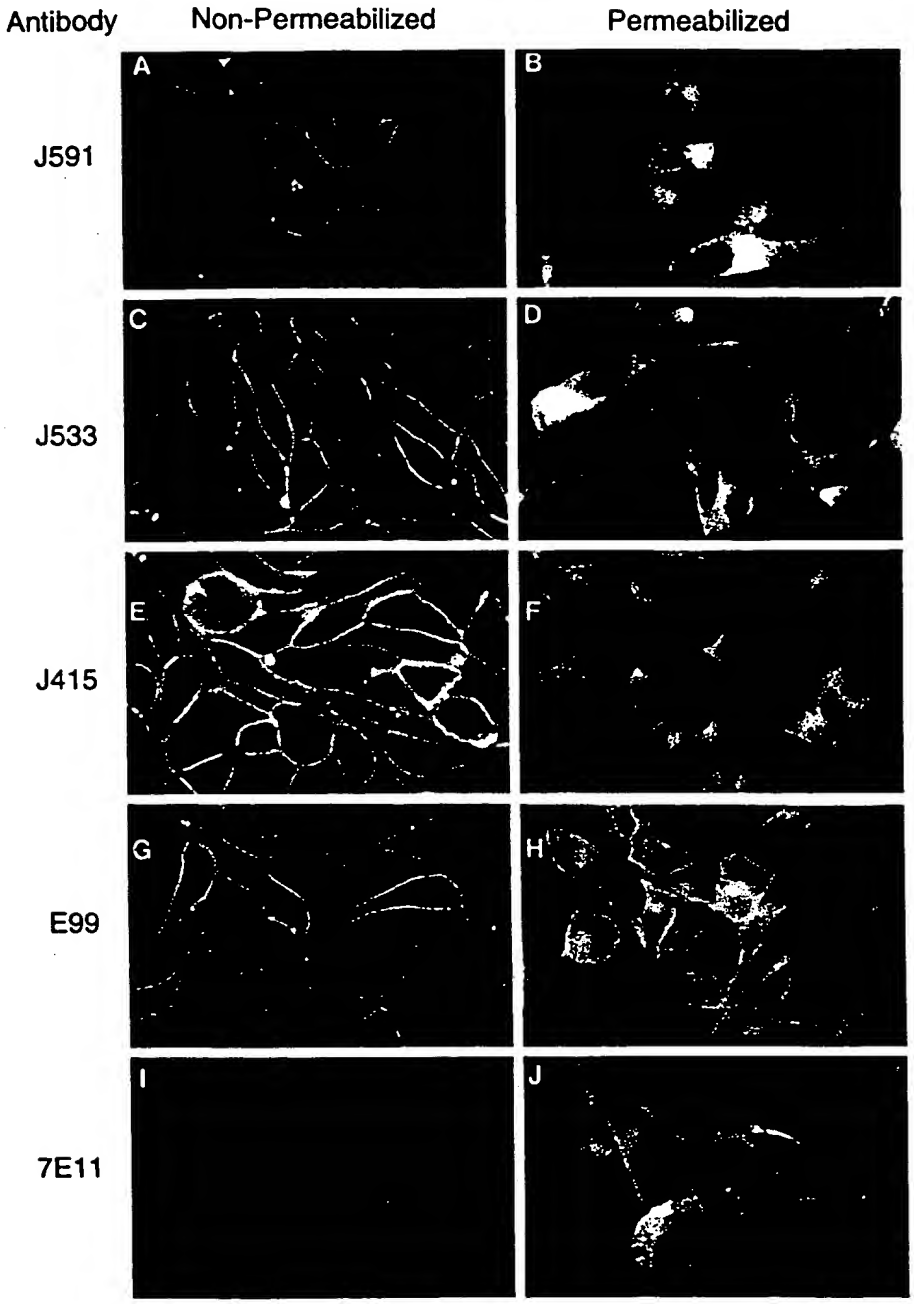


Fig. 3. Immunofluorescence assay comparing the reactivity of mAbs J591, J415, J533, and E99 to 7E11 on nonpermeabilized and permeabilized LNCaP cells. Intact, nonpermeabilized cells are reactive with mAbs J591 (*A*), J533 (*C*), J415 (*E*), and E99 (*G*) but not 7E11 (*I*). Reactivity is limited to the cell membrane without cytoplasmic staining, as mAbs do not enter the intact cells. Failure of 7E11 to bind (*I*) is consistent with the intracellular location of its epitope. When the cells are permeabilized prior to mAb incubation (*B, D, F, H, J*), reactivity to both cytoplasmic and membrane PSMA is seen. After the permeabilization and exposure of the intracellular PSMA epitope, 7E11 does bind. ×1500.

ing, substrate (para nitrophenylphosphate) was added, and reactivity was read at $A_{405\ nm}$. Irrelevant antibody (156) was used as a control.

## Results

From over 2000 clones screened, 4 clones that reacted with a 100-kDa band on Western blots and that produced strong immunohistochemical staining of prostate epithelium were selected for further characterization.

**Immunoprecipitation/Immunoblot.** In Western blot analysis, mAbs J591 (IgG1), J533 (IgG1), J415 (IgG1), and E99 (IgG3), as well as 7E11, identified a 100-kDa band from LNCaP lysate but not from the PSMA-negative PC3 lysate (data not shown). To confirm that mAbs J591, J533, J415, and E99 detected the same antigen as 7E11, a cross-immunoprecipitation experiment was performed. Fig. 1 illustrates that the 100-kDa band that was immunoprecipitated by mAbs J591, J533, J415, E99, or 7E11 was detectable by immunoblot using either J591 or 7E11 as a probe (Fig. 1, *A* and *B*, respectively). Sequential immunoprecipitation studies (data not shown) also demonstrated that 7E11 and the four new mAbs can preclear reactivity to one another.

**Immunohistochemical Reactivity.** The reactivity of mAbs J591, J533, J415, and E99 with normal human tissues and cancers, with rare exception (*vide infra*), were similar to 7E11. Normal tissues with similar immunohistochemical reactivity included prostate (normal and hyperplastic glands demonstrated heterogeneous, weak to moderate staining intensity), kidney (subset of proximal

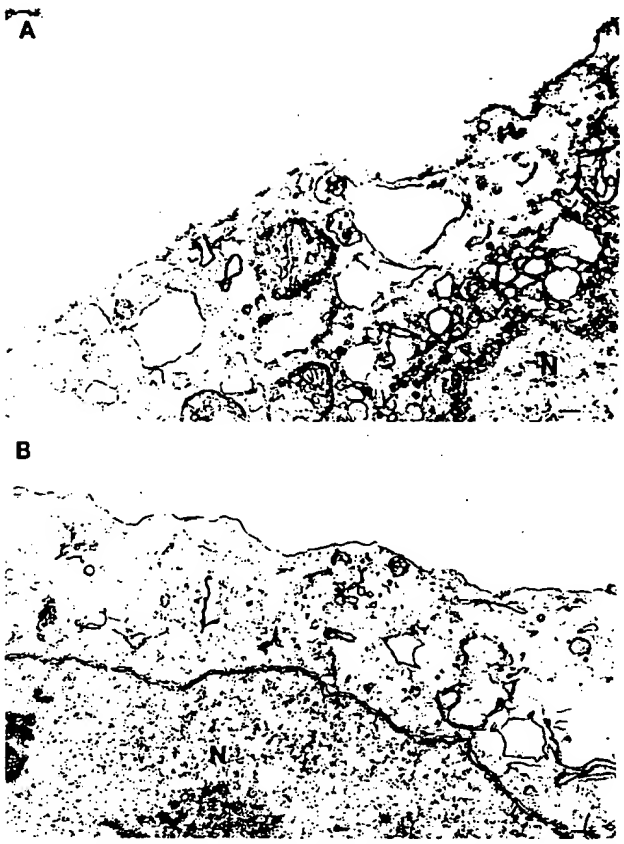


Fig. 4. IEM showing the reactivity of mAbs J591 (*A*) and 7E11 (*B*) to viable LNCaP cells. mAb J591 localizes to the extracellular surface of the plasma membrane, whereas 7E11 demonstrates no binding. *Bar*, 0.6 $\mu$ (*A*) and 1.0 $\mu$ (*B*). *N*, nucleus.

tubules), and duodenum (weakly reactive). The only normal tissue in which we found any difference in reactivity was striated muscle. Although 7E11 was strongly reactive to striated muscle, mAbs J591, J533, J415, and E99 demonstrated no reactivity. In neoplastic tissues, findings were again similar when comparing 7E11 to mAbs J591, J533, J415, and E99. All 21 PCas studied were strongly reactive with mAbs J591, J533, J415, and E99, being somewhat more intense and more homogeneous than 7E11. As reported previously (17), we found 7E11 reacted with vascular endothelium in a subset of tumors. However, mAbs J591, J533, J415, and E99 reacted more strongly with vascular endothelium in all 23 carcinomas studied (Fig. 2), including 9 of 9 renal, 5 of 5 urothelial, 6 of 6 colon, 1 of 1 lung, 1 of 1 breast and 1 of 1 metastatic adenocarcinoma to the liver.

**Immunofluorescence Staining of LNCaP Cells.** We compared, by indirect immunofluorescence, mAbs J591, J533, J415, and E99 to mAb 7E11 on viable or fixed, permeabilized or nonpermeabilized LNCaP cells (Fig. 3). LNCaP cells with intact plasma membrane (*i.e.*, either viable [data not shown] or fixed without permeabilization) demonstrated cell surface reactivity with mAbs J591, J533, J415, and E99 (Fig. 3, *A-C, E, G*), but not with mAb 7E11 (Fig. 3*I*). Only after LNCaP cells were permeabilized could 7E11 reactivity be demonstrated (Fig. 3*J*). Once permeabilized, the reactivity of all mAbs appeared both in the cytoplasm and on the plasma membrane.

**Immunoelectron Microscopy.** IEM similarly demonstrated immunoreactivity of mAb J591 (Fig. 4*A*) but not 7E11 (Fig. 4*B*) with viable LNCaP cells. Furthermore, the IEM photomicrographs of mAb J591 show the gold particles localized to the extracellular face of the plasma membrane, confirming reactivity with the extracellular domain of PSMA.

**Competitive Binding Assay.** A double antibody sandwich competition ELISA was used to determine whether the four mAbs recognize the same or different epitopes (Fig. 5). Each unlabeled mAb was able to block its biotinylated counterpart serving as a positive control. An unrelated IgG1 antibody (I56) did not block any of the mAbs to PSMA. J591, J533, and E99 were each able to block each other, but were not blocked by J415. Conversely, J415 was blocked only by its unlabeled counterpart but not by any of the other three mAbs. These results indicate that J591, J533, and E99 recognize the same epitope that is distinct and noncross-reactive with the epitope recognized by J415.

## Discussion

This study defines four new IgG mAbs that detect two distinct extracellular epitopes of PSMA ($PSMA_{ext1}$ and $PSMA_{ext2}$). The reactivity of these mAbs with PSMA has been defined by immunoprecipitation and immunoblotting studies and reactivity against cell lines (data not shown) and tissue sections using the 7E11 mAb as a reference. Immunoprecipitation and immunoblotting studies demonstrate identical reactivity to that seen with 7E11. Reactivity *in vitro* (data not shown) and on tissue sections of normal and neoplastic specimens demonstrates nearly identical results. The exceptions in immunohistochemical reactivity were limited to striated muscle and tumor vascular endothelium. Striated muscle is reactive with 7E11 but not with mAbs J591, J415, J533, or E99. 7E11 reactivity with striated muscle had been reported previously by Lopes *et al.* (4) who, like the present study, used frozen sections but has been reported as negative by Silver *et al.* (17) who studied paraffin sections. This discrepancy is most likely explained by some loss of 7E11/PSMA immunoreactivity in the fixation/embedding process. The difference in reactivity of 7E11 and the present mAbs to striated muscle, both herein studied on frozen sections, may represent differences in the posttranslational processing of PSMA (the external domain of which is heavily glycosylated) occurring in prostate as compared to muscle.

Reactivity of 7E11 with tumor but not normal vascular endothelium also was noted previously by Silver *et al.* (17), although 7E11 reactivity was reported in only half of their renal and urothelial cancers (15 of 30) and 3 of 19 colon carcinomas. In the present study, mAbs J591, J415, J533, and E99 demonstrate reactivity with tumor vasculature in all 23 nonprostate carcinomas tested. Some of the increased reactivity seen herein may represent the benefit of studying frozen as compared to paraffin sections. Within this study, when comparing mAbs J591, J415, J533, and E99 to 7E11 using a constant tissue preparation (frozen sections), we found stronger reactivity with mAbs J591, J415, J533, and E99 than with 7E11. The most likely explanation for the generally stronger reactivity seen with these new mAbs is that they were selected for, among other features, strong immunohistochemical reactivity. We have not studied the immunohistochemical reactivity of mAbs J591, J415, J533, and E99 on paraffin sections.

The initial study with 7E11 (1) indicated reactivity to fixed, but not viable, LNCaP cells later explained by epitope mapping studies indicating the 7E11 epitope to be intracellular (12). A more recent study by Troyer *et al.* (13) studying ultrathin sections by IEM demonstrated 7E11 reactivity on the cytoplasmic aspect of LNCaP

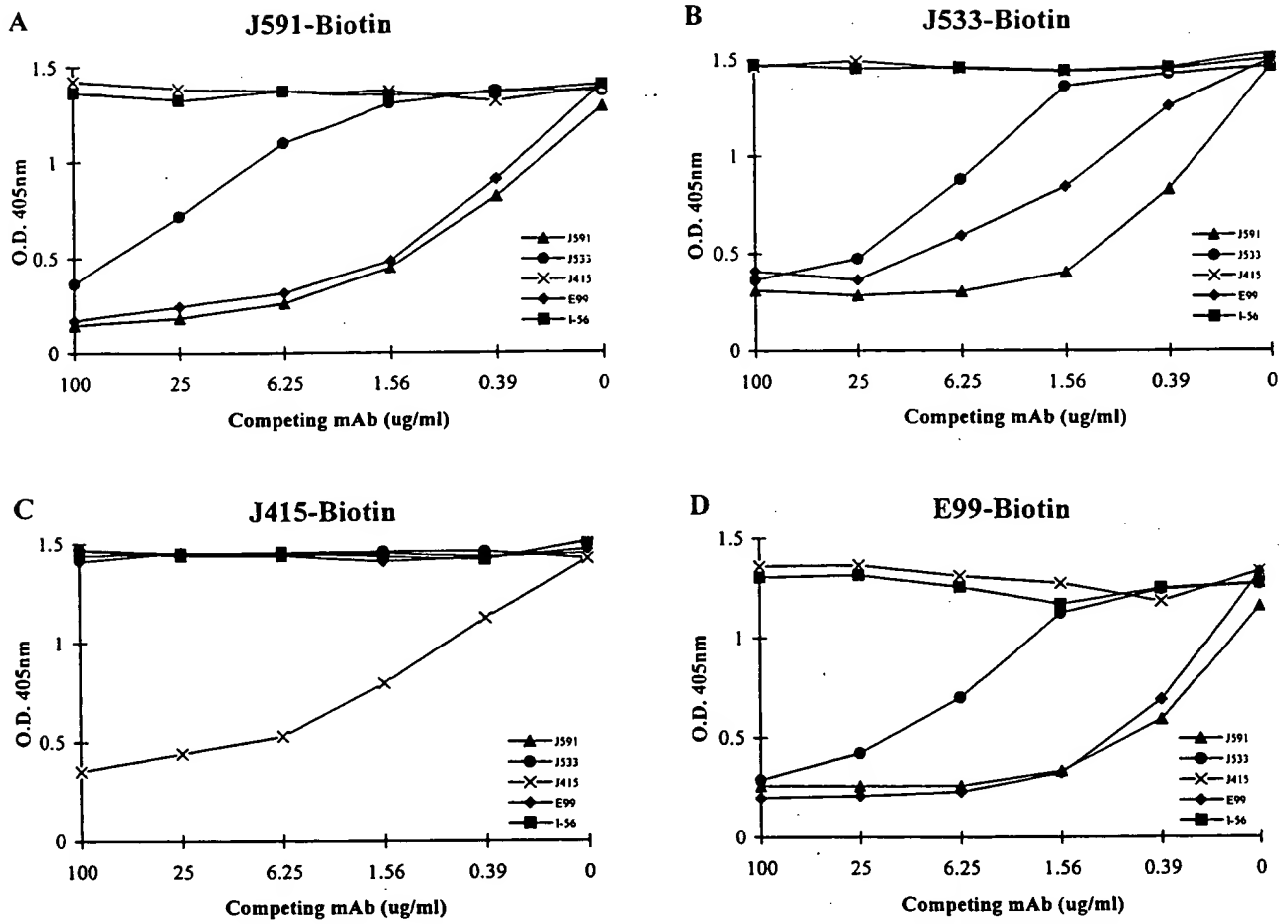


Fig. 5. Competitive binding assay of biotinylated mAbs. PSMA was first captured by 7E11, then unlabeled blocking mAb was added, followed 1 h later by the indicated biotinylated mAb: 0.3 μg/ml J591-biotin (*A*); 1.25 μg/ml J533-biotin (*B*); 0.2 μg/ml J415-biotin (*C*); and 1.25 μg/ml E99-biotin (*D*). Each unlabeled mAb blocks its biotinylated counterpart (positive control); mAb I56 (to PSP) did not block (negative control). mAbs J591, J533, and E99 compete with each other for binding, whereas J415 does not.

cells plasma membrane. Troyer *et al.* (13) also confirmed 7E11 reactivity with permeabilized but not with nonpermeabilized LNCaP cells. Our studies comparing 7E11 with the present mAbs by immunofluorescence assays on viable and fixed, permeabilized and nonpermeabilized LNCaP cells confirmed the data published previously that 7E11 detects an intracellular epitope not available for mAb binding unless the cell membrane is disrupted. A recent report by Barren *et al.* (18) represents the sole study indicating that 7E11 can react with viable LNCaP cells. The report by Barren *et al.* is inconsistent with other published work (1, 12, 13), as well as the results reported here, and may be due to a technical point. Barren *et al.*, after incubating 7E11 with viable LNCaP cells, harvested LNCaP for flow cytometry by scraping the cells in the presence of 7E11. As scraping can disrupt cell membranes, this would have provided 7E11 access to its intracellular epitope, which likely accounts for the reactivity reported. Importantly, mAbs J591, J415, J533, and E99, unlike 7E11, can bind to either viable or nonpermeabilized cells consistent with targeting accessible epitopes on the extracellular domain of PSMA. Our IEM finding of mAb J591 localization on the extracellular aspect of the plasma membrane (Fig. 4*A*), in contrast to the intracellular localization of 7E11 on IEM reported by Troyer *et al.* (13), provides further evidence of reactivity of the present mAbs to the extracellular domain of PSMA.

Epitope mapping of the four present mAbs demonstrates that J591, J533, and E99 each bind to a single epitope ($PSMA_{ext1}$), whereas J415 binds to a different, noncompeting site ($PSMA_{ext2}$). This will allow the development of a "sandwich" assay to determine the presence and measure the level of PSMA in serum, which is an area of some current controversy (14, 16).

By allowing the study of viable cells, these mAbs will be useful for studies of PSMA function and PCa cell biology. Recent work indicates that PSMA has glutaminase (19, 20) activity. Studies are under way to determine whether mAbs to $PSMA_{ext1}$ and/or $PSMA_{ext2}$ can block this enzymatic activity and, if so, the effect of such a blockade on normal and neoplastic prostate physiology.

Given prior understanding of PSMA specificity and expression and the established ability of 7E11/CYT-356 to localize *in vivo* to a substantially less available epitope, one would anticipate the likelihood that these new mAbs might demonstrate significantly improved *in vivo* targeting for imaging and therapy. The immunoreactivity of these mAbs to vascular endothelium of a wide variety of cancers may significantly broaden their *in vivo* utility.

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# Five Different Anti-Prostate-specific Membrane Antigen (PSMA) Antibodies Confirm PSMA Expression in Tumor-associated Neovasculature[1]

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## ABSTRACT

Prostate-specific membrane antigen (PSMA) is a type II integral membrane glycoprotein that was initially characterized by the monoclonal antibody (mAb) 7E11. PSMA is highly expressed in prostate secretory-acinar epithelium and prostate cancer as well as in several extraprostatic tissues. Recent evidence suggests that PSMA is also expressed in tumor-associated neovasculature. We examined the immunohistochemical characteristics of 7E11 and those of four recently developed anti-PSMA mAbs (J591, J415, and Hybritech PEQ226.5 and PM2J004.5), each of which binds a distinct epitope of PSMA. Using the streptavidin-biotin method, we evaluated these mAbs in viable prostate cancer cell lines and various fresh-frozen benign and malignant tissue specimens. In the latter, we compared the localization of the anti-PSMA mAbs to that of the anti-endothelial cell mAb CD34. With rare exceptions, all five anti-PSMA mAbs reacted strongly with the neovasculature of a wide spectrum of malignant neoplasms: conventional (clear cell) renal carcinoma (11 of 11 cases), transitional cell carcinoma of the urinary bladder (6 of 6 cases), testicular embryonal carcinoma (1 of 1 case), colonic adenocarcinoma (5 of 5 cases), neuroendocrine carcinoma (5 of 5 cases), glioblastoma multiforme (1 of 1 cases), malignant melanoma (5 of 5 cases), pancreatic duct carcinoma (4 of 4 cases), non-small cell lung carcinoma (5 of 5 cases), soft tissue sarcoma (5 of 6 cases), breast carcinoma (5 of 6 cases), and prostatic adenocarcinoma (2 of 12 cases). Localization of the anti-PSMA mAbs to tumor-associated neovasculature was confirmed by CD34 immunohistochemistry in sequential tissue sections. Normal vascular endothelium in non-cancer-bearing tissue was consistently PSMA negative. The anti-PSMA mAbs reacted with the neoplastic cells of prostatic adenocarcinoma (12 of 12 cases) but not with the neoplastic cells of any other tumor type, including those of benign and malignant vascular tumors (0 of 3 hemangiomas, 0 of 1 hemangioendothelioma, and 0 of 1 angiosarcoma). The mAbs to the extracellular PSMA domain (J591, J415, and Hybritech PEQ226.5) bound viable prostate cancer cells (LNCaP and PC3-PIP), whereas the mAbs to the intracellular domain (7E11 and Hybritech PM2J004.5) did not. All five anti-PSMA mAbs reacted with fresh-frozen benign prostate secretory-acinar epithelium (28 of 28 cases), duodenal columnar (brush border) epithelium (11 of 11 cases), proximal renal tubular epithelium (5 of 5 cases), colonic ganglion cells (1 of 12 cases), and benign breast epithelium (8 of 8 cases). A subset of skeletal muscle cells was positive with 7E11 (7 of 7 cases) and negative with the other four anti-PSMA mAbs. PSMA was consistently expressed in the neovasculature of a wide variety of malignant neoplasms and may be an effective target for mAb-based antineovasculature therapy.

## INTRODUCTION

PSMA[3] is a type II membrane glycoprotein of $M_r$ ~100,000 that was initially characterized by the mAb 7E11 (1, 2). Recent studies have confirmed the location of the PSMA gene on chromosome 11p and have demonstrated the existence of a related PSMA-like gene on 11q (3–5). Two variant forms of PSMA, initially predicted to exist as PSMA, and a spliced form, PSM′, have been subsequently confirmed. PSMA is highly expressed in benign prostate secretory-acinar epithelium, prostatic intraepithelial neoplasia, and prostatic adenocarcinoma (2, 6–8), and evidence suggests that PSMA expression is greatest in high-grade and hormone-insensitive cancers (2, 9–11). A shorter, alternatively spliced and presumably cytosolic form of PSMA, named PSM′, is the predominant form expressed in benign prostate epithelium (12, 13). Several studies have shown that anti-PSMA mAbs bind to several nonprostate tissues, including duodenum and kidney (6, 14, 15), and to the vasculature associated with solid malignant tumors (15, 16).

The function of PSMA is currently under investigation. Pinto *et al.* (17) demonstrated that PSMA has a folate hydrolase-type of activity because LNCaP cells were shown to hydrolyze γ-glutamyl linkages in methotrexate triglutamate. Others have demonstrated that PSMA has a neuropeptidase-type function (18, 19). On the basis of these enzymatic characteristics, the nomenclature committee of the International Union of Biochemistry and Molecular Biology has recommended for PSMA the formal name of glutamate carboxypeptidase (EC 3.4.17.21; Ref. 20).

The 7E11 antibody is a specific murine IgG mAb that was derived after immunization of mice with preparations from the LNCaP human prostate cancer cell line (1). 7E11 has been well characterized and is known to bind an intracellular epitope of PSMA not present on PSM′. As a result, 7E11 does not bind viable prostate cancer cells (1, 16, 21). Modified by the addition of $^{111}$In, 7E11 is used currently at some centers as an imaging agent *in vivo*. Clinical trials have demonstrated that this radioimmunoconjugate of 7E11, known as $^{111}$In-capromab pendetide, may be a useful adjunct in identifying and localizing metastatic or recurrent prostate cancer (22–25).

A number of other anti-PSMA mAbs have been developed recently that bind epitopes that are distinct from that recognized by 7E11 (13, 16). For example, the mAbs J591, J415, J533, and E99 bind to the extracellular PSMA domain (16). Investigators at Hybritech Inc. (San Diego, CA) have identified and purified the mAb PEQ226.5, which binds the peptide backbone of the PSMA extracellular domain. In addition, investigators at Hybritech Inc. have identified PM2J004.5, which binds an epitope of the intracellular PSMA domain that is distinct from that bound by 7E11 (13).

The purpose of this study was to compare the immunohistochemical profiles of four recently developed anti-PSMA mAb to that of 7E11. Specifically, we evaluated these mAbs in prostate cancer cell lines, benign and malignant prostate tissue, benign nonprostate tissue,

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[3] The abbreviations used are: PSMA, prostate-specific membrane antigen; mAb, monoclonal antibody; OC, organ-confined.

and a variety of malignant tissues. In the latter, we sought further to confirm PSMA expression in tumor-associated neovasculature.

## MATERIALS AND METHODS

**Tissue Specimens and Antibodies.** The LNCaP, PC3, and PC3-PIP (PC3 cells transfected with PSMA[4]) were obtained from cell lines cultured in the George M. O'Brien Urology Research Center at Memorial Sloan-Kettering Cancer Center. Fresh-frozen tissue samples from male and female patients were randomly obtained from the Memorial Sloan-Kettering Cancer Center institutional tissue bank. Twenty different benign tissue types, including prostate tissue, were examined, as were the following tumor types: conventional (clear cell) renal cell carcinomas, transitional cell carcinomas of the urinary bladder, testicular-embryonal carcinoma, colonic adenocarcinomas, neuroendocrine carcinomas, glioblastoma multiforme, malignant melanomas, pancreatic duct carcinomas, non-small cell lung carcinomas, soft tissue sarcomas, benign and malignant vascular tumors, breast carcinomas, and prostatic adenocarcinomas. The 7E11 mAb was provided by Cytogen, Inc. (Princeton, NJ). The J591 and J415 antibodies were recently developed, and their characteristics were demonstrated previously (16). The mAbs PEQ226.5 and PM2J004.5 were provided by Hybritech Inc. (San Diego, CA) and also described previously (13). The anti-endothelial cell mAb CD34 (Immunotech, Coulter Company, Opa Locka, FL) was used for comparative immunohistochemical reactions in all cancerous tissue types.

**Immunohistochemistry.** LNCaP, PC3, and PC3-PIP were grown in cell culture wells to ~80% confluence. Immunohistochemical studies were then performed on the different cell types in either a viable or a fixed state. For fixation, the cells were treated with 10% buffered formalin for 10 min. The cells were then incubated with the different mAbs at 5 μg/ml at room temperature for 45 min. For live cells, after incubation with the primary antibody under the same conditions, the cells were then fixed in cold 10% buffered formalin for 10 min. The immunohistochemical reaction was completed by the streptavidin-biotin method. Briefly, the sections were washed thoroughly in 1.0% PBS, and biotinylated secondary antibody, horse anti-mouse IgG, was added for 60 min. After washing with PBS, streptavidin was added to the specimens for 60 min, and the slides were washed again in PBS. Next, the specimens were immersed for 5 min in a fresh solution of 0.06% diaminobenzidine tetrachloride and 0.01% hydrogen peroxide. Following washing, the sections were counterstained with hematoxylin, dehydrated, and mounted.

Tissue samples were snap-frozen in OCT compound placed in isopentane and stored at −70°C. Multiple 5-μm cryostat tissue sections were then cut and fixed in cold acetone (4°C) for 12 min. Prior to primary mAb incubation, the specimens underwent 30-min incubation with a normal horse blocking serum 1:20 in 2.0% BSA. The primary antibody incubations (5 μg/ml) were then performed with 7E11, J591, J415, PEQ226.5, PM2J004.5, and CD34 (in the cancer cases) for 60 min at room temperature. The remainder of the immunohistochemical reaction was completed using the streptavidin-biotin method as described previously. In tissue with known significant quantities of endogenous biotin, the immunoperoxidase method was used with rabbit antimouse immunoglobulin-peroxidase as the secondary antibody (Envision; DAKO Corp., Carpinteria, CA). In all tissue sections, negative controls were performed using blocking serum in place of the primary antibody. The immunohistochemical reactivities of all of the mAbs were then evaluated and compared.

## RESULTS

**Tumor-associated Neovasculature.** With rare exceptions, all five anti-PSMA mAbs bound tumor-associated neovasculature of nonprostatic tumors (Table 1 and Fig. 1). The neovasculature of one breast carcinoma and one soft tissue sarcoma (myxofibrosarcoma) showed no immunoreactivity; however, both contained CD34-positive vasculature. The four cases of breast carcinoma with PSMA-positive neovasculature were ductal carcinomas, and the one PSMA-negative case was lobular carcinoma. Interestingly, only a small subset of prostate cancer specimens showed PSMA-positive neovasculature (2 of 12 cases). In these cases, we found the CD34-stained sections to be useful in localizing so-called "hot spots" of neovasculature that we then compared to the anti-PSMA mAb-stained sections. This helped us confirm the location of vessels amid strongly PSMA-positive tumor cells. We noted no significant histological differences between prostate cancers with PSMA-positive neovasculature and those with PSMA-negative neovasculature. In all of the tumors, 7E11, J591, J415, PEQ226.5, and PM2J004.5 mAbs bound neovasculature in a like manner (Fig. 2). The results of CD34 immunohistochemistry in sequential tissue sections confirmed localization of the anti-PSMA mAbs to neovasculature endothelium (Fig. 2). In contrast to tumor-associated neovasculature, none of the anti-PSMA mAbs reacted with vasculature in the non-cancer-bearing tissue sections. The staining intensity of the external domain-binding mAbs (J591, J415, and PEQ226.5) in tumor-associated neovasculature was greater than that of the internal domain-binding mAbs (7E11 and PM2J004.5).

Table 1 *Results of PSMA immunohistochemistry in tumor cells and tumor-associated neovasculture*

| Tumor | No. of positive tumors/total no. of tumors studied | |
|---|---|---|
| | Tumor cells | Neovasculature |
| Conventional renal cell carcinoma | 0/11 | 11/11 |
| Transitional cell carcinoma | 0/6 | 6/6 |
| Testicular embryonal carcinoma | 0/1 | 1/1 |
| Colonic adenocarcinoma | 0/5 | 5/5 |
| Neuroendocrine carcinoma | 0/5 | 5/5 |
| Glioblastoma multiforme | 0/1 | 1/1 |
| Malignant melanoma | 0/5 | 5/5 |
| Pancreatic duct carcinoma | 0/4 | 4/4 |
| Non-small cell lung carcinoma | 0/5 | 5/5 |
| Soft tissue sarcoma | 0/6 | 5/6 |
| Breast carcinoma | 0/6 | 5/6 |
| Hemangioma | 0/3 | 0/3 |
| Hemangioendothelioma | 0/1 | 0/1 |
| Angiosarcoma | 0/1 | 0/1 |
| Angiolipoma | 0/1 | 0/1 |
| Angiomyolipoma | 0/2 | 0/2 |
| Prostatic adenocarcinoma | 12/12 | 2/12 |

**Malignant Tumor Cells.** All 12 prostate cancer cases were strongly PSMA positive, and all nonprostate tumor cells were PSMA negative (Table 1). All vascular tumors were CD34 positive but PSMA negative.

**Prostate Cancer Cell Lines.** The external domain-binding mAbs (J591, J415, and PEQ226.5) bound viable LNCaP and PC3-PIP cells that are known to express PSMA. In contrast, the internal domain-binding mAbs (7E11 and PM2J004.5) did not bind viable LNCaP and PC3-PIP cells (Fig. 3). After formalin fixation, all anti-PSMA mAbs, including 7E11 and PM2J004.5, reacted with LNCaP and PC3-PIP cells. None of the mAbs bound viable or formalin-fixed PC3 cells that are known to lack PSMA expression.

**Benign Tissues.** Although benign prostatic secretory-acinar epithelium displayed heterogeneous staining with the five mAbs, all 28 benign prostate cases were PSMA positive. Immunoreactivity was typically concentrated at the luminal aspect of the cytoplasmic membrane. Basal epithelium and stromal cells were PSMA negative. The immunoreactivity of the benign secretory-acinar epithelium was less intense than that of prostatic adenocarcinoma, and the staining intensity of the external domain-binding mAbs J591, J415, and PEQ226.5 was greater than that of the internal domain-binding mAbs 7E11 and PM2J004.5 (data not shown).

The anti-PSMA mAbs reacted with several of the 19 benign nonprostate tissues (Table 2). All five mAbs reacted with duodenal

[4] J. B. Latouche and M. Sadelain, unpublished observations.

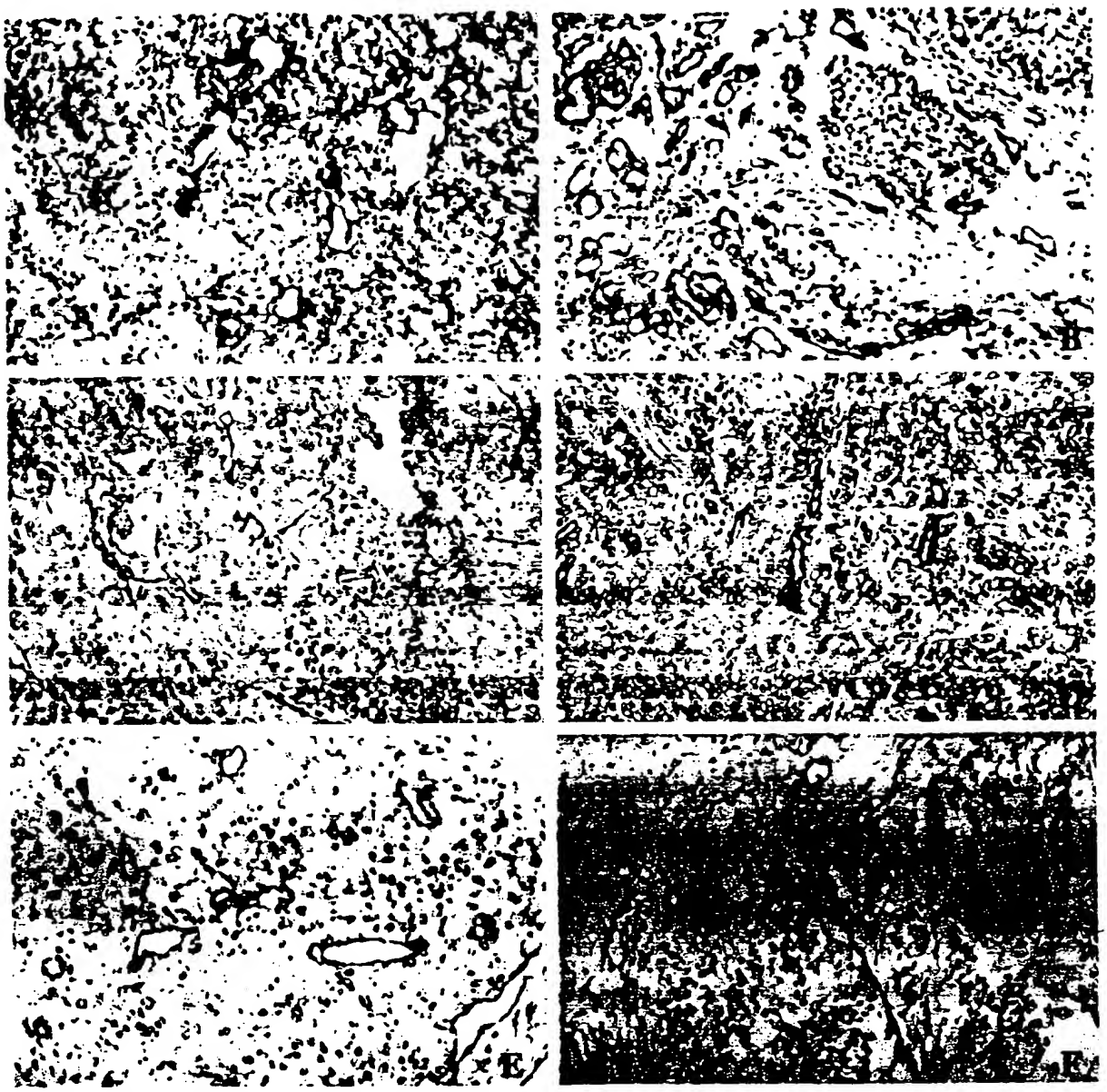

Fig. 1. PSMA expression in tumor-associated neovasculature. Immunohistochemical reactivity with external domain-binding anti-PSMA mAbs J591 or PEQ226.5 in representative cancer types. *A*, J591, breast cancer; *B*, PEQ226.5, transitional cell carcinoma of the urinary bladder; *C*, J591, malignant melanoma; *D*, PEQ226.5, non-small cell lung carcinoma; *E*, J591, soft tissue sarcoma; and *F*, J591, neuroendocrine carcinoma.

columnar (brush border) epithelium (11 of 11 cases), renal proximal tubular epithelium (5 of 5 cases), benign breast epithelium (8 of 8 cases), and colonic ganglion cells (1 of 12 cases). In skeletal muscle, a subset of muscle fibers were positive only with 7E11 and negative with the other four mAbs (Fig. 4). The vasculature in all benign tissues was uniformly PSMA negative. The staining intensity of these PSMA-positive benign tissues was less than that of prostate cancer and tumor-associated neovasculature.

## DISCUSSION

Our study confirms PSMA expression in the neovasculature of a wide spectrum of malignant neoplasms. Specifically, we found PSMA expression in various epithelial tumors (carcinomas), neuroendocrine tumors, and mesenchymal tumors (soft tissue sarcomas) and in malignant melanoma and glioma. In contrast to previous studies, we used five anti-PSMA mAbs, each of which binds a different epitope of the intracellular or extracellular PSMA domain. Thus, our results provide further evidence that PSMA, rather than a PSMA-like molecule, is expressed in tumor-associated neovasculature. Also in contrast to previous studies, we confirmed localization of PSMA to endothelial cells with the mAb CD34, an anti-endothelial cell marker used to study angiogenesis and determine microvessel density (26–30).

Our findings are consistent with previous studies showing PSMA expression in tumor-associated neovasculature. For example, Silver *et al.* (15) demonstrated 7E11 binding and "neoexpression of PSMA in endothelial cells" in a subset of tumors, including renal cell carcinoma (unspecified type), transitional cell carcinoma of the urinary bladder, and colonic adenocarcinoma. More recently, Liu *et al.* (16) studied

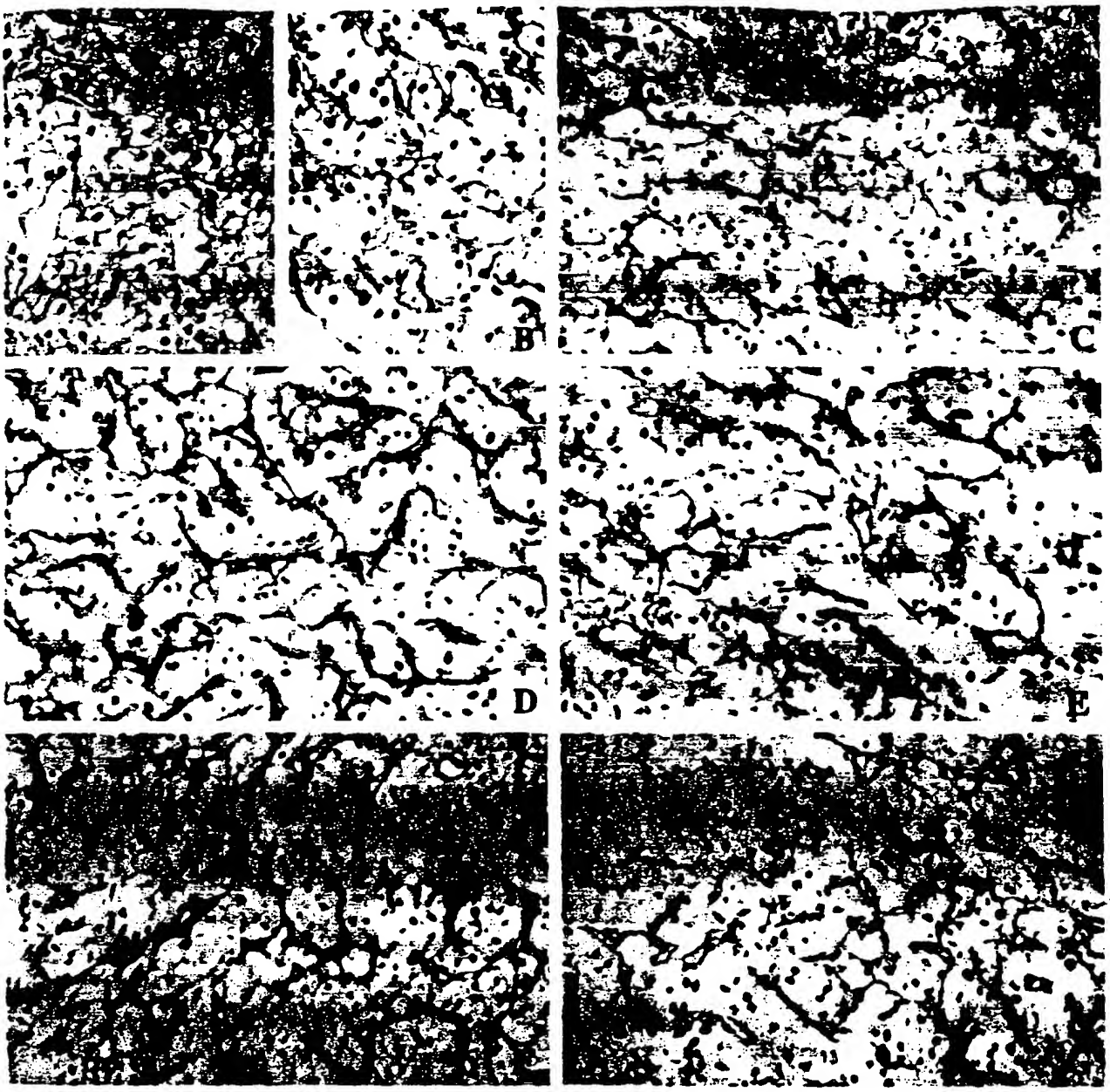


Fig. 2. Binding profile of the five anti-PSMA mAbs and CD34 in the neovasculature of conventional (clear cell) renal cell carcinoma. *A*, H&E-stained section; *B*, CD34; *C*, 7E11; *D*, J591; *E*, J415; *F*, PEQ226.5; and *G*, PM2J004.5.

four external domain-binding anti-PSMA mAbs (J591, J415, J533, and E99) and showed that each bound the tumor-associated neovasculature in several nonprostatic carcinomas. Although it is unclear whether PSMA is produced by endothelial cells of tumor-associated neovasculature or whether it is produced in other tissues and sequestered from the serum, we favor the former because PSMA is expressed only in a limited number of benign tissues and in prostate cancer but is not expressed in other malignant cell type. In addition, circulating PSMA has not been demonstrated in serum.[5] Additional studies, however, are necessary to confirm this hypothesis.

We found that endothelial cell expression of PSMA was restricted to the neovasculature of malignant neoplasms. In fact, neither the vascular endothelial cells of benign tissues nor the neoplastic cells of vascular tumors expressed PSMA. These results suggest that endothelial cell-PSMA expression may be stimulated by one or more tumor-secreted angiogenic factors. The fact that all of the vascular neoplasms we studied, including the one example of angiosarcoma, were PSMA negative is not surprising, given that, in these tumors, the endothelium itself is neoplastic and, presumably, not stimulated by angiogenic factors. The presence or absence of PSMA expression in benign neovasculature (*e.g.*, granulation tissue, endometrium, and so on) remains to be established.

The neovasculature associated with OC prostatic adenocarcinoma only rarely expressed PSMA. Others also have found no detectable

[5] H. Liu and N. H. Bander, unpublished observations.

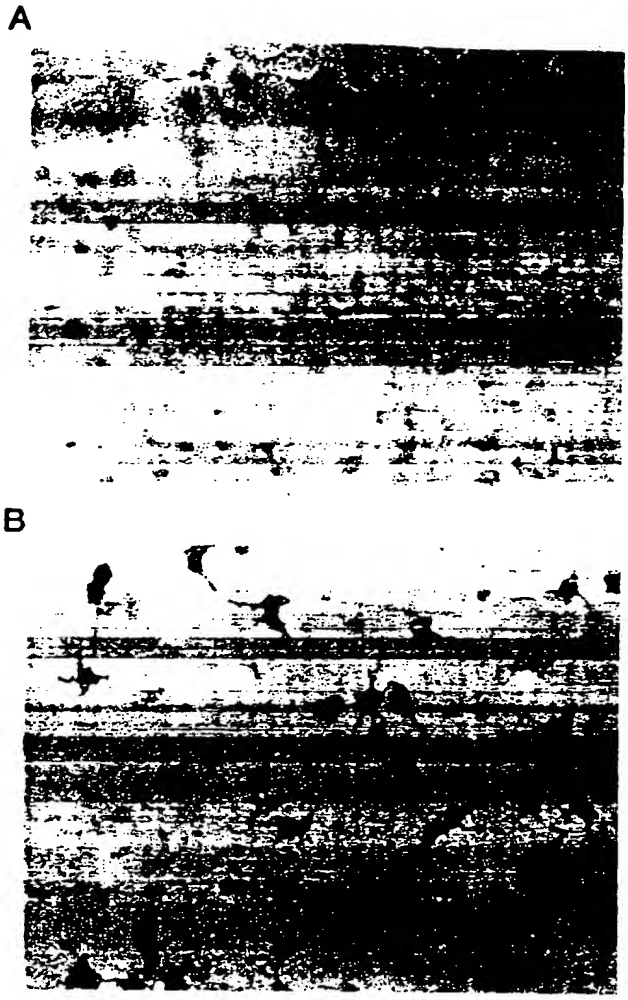


Fig. 3. Comparative immunohistochemistry in viable PSMA-expressing PC3-PIP cells. *A*, 7E11 demonstrating no immunoreactivity. *B*, J591 demonstrating positive immunoreactivity with live cells.

PSMA expression in OC prostate cancer-associated neovasculature (9, 15). These observations are remarkable given the ubiquity of PSMA expression in tumor-associated neovasculature of other cancer types. They are, however, not altogether surprising, given the histological features of OC prostate cancer. For example, in contrast to many other epithelial tumors such as ductal carcinoma of the breast or pancreas, OC prostate cancer typically is not associated with an exuberant host-stromal reaction. Lobular carcinoma of the breast, like prostatic adenocarcinoma, typically does not induce a marked desmoplastic stromal response. Interestingly, the one breast cancer specimen in our series with PSMA-negative neovasculature was an example of lobular carcinoma. These results suggest that PSMA expression in tumor-associated neovasculature may be related to the degree and nature of neoangiogenesis. The relationship between primary tumor stage in different malignancies and PSMA expression in neovasculature is unknown.

Consistent with most previous studies, we found that mAbs to the intracellular PSMA domain (7E11 and PM2J004.5) do not bind viable prostate cancer cells, whereas mAbs to the external domain (J591, J415, and PEQ226.5) do bind live cells (16, 21). Only one study has reported 7E11 binding with viable prostate cancer cells (31). It is postulated that 7E11 binds predominantly to apoptotic cells within prostate cancer sites *in vivo*. Apoptotic cells, unfortunately, comprise only a minority of the total prostate tumor-cell population. This, no doubt, has contributed to the relatively low sensitivity of $^{111}$In-capromab pendetide as an imaging agent for prostate cancer. In this regard, targeting the extracellular PSMA domain with radioimmunoconjugates may enhance prostate cancer cell labeling *in vivo*.

The results of several but not all immunohistochemical studies using the 7E11 mAb have shown that PSMA is expressed in a limited number of nonprostatic tissues (1, 6, 15). Our findings support the results of other studies showing PSMA expression in duodenal (brush border) epithelium and renal proximal tubular epithelium but suggest that PSMA expression in these tissues is less than it is in prostate cancer and tumor-associated neovasculature (15, 16). Duodenal brush-border epithelium has high levels of folate hydrolase activity that is essential for folate absorption (17). This folate hydrolase activity is localized to the luminal membrane and is consistent with the staining pattern of the anti-PSMA mAbs. Proximal renal tubular epithelium also actively reabsorbs folate through the luminal membrane (32). Halsted *et al.* (33) found significant sequence homology between pig intestinal folate hydrolase (folypoly-gamma-glutamate carboxypeptidase) and human PSMA, suggesting that human duodenal membrane folate hydrolase may represent PSMA. Alternatively, it may represent a closely related enzyme that cross-reacts with anti-PSMA mAbs. In contrast to previous studies, we found consistent PSMA expression in mammary ductal epithelium. The reasons for our conflicting results are unclear; however, previous studies showing no PSMA expression in breast may have included specimens with inadequate amounts of ductal epithelium. One of our 12 colon specimens displayed PSMA expression in ganglion cells. The relatively sparse immunoreactivity observed in colonic ganglia may be indicative of peripheral neuronal PSMA expression previously described in nonmyelinating, perisynaptic Schwann cells near motoneuron terminal endplates (34).

The staining profile of skeletal muscle is unique, in that a subset of cells is positive with only 7E11. Liu *et al.* (16) also showed a subset of skeletal muscle cells bind 7E11 and not other anti-PSMA mAbs. Of

Table 2 *Results of PSMA immunohistochemistry using five different anti-PSMA mAbs in fresh-frozen benign tissue*

| Tissue | No. of positive cases/total no. of cases studied | | | | |
|---|---|---|---|---|---|
| | 7E11 | J591 | J415 | PEQ226.5 | PM2J004.5 |
| Prostate | 28/28 | 28/28 | 28/28 | 28/28 | 28/28 |
| Lung | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 |
| Brain | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| Digestive system | | | | | |
| Parotid | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 |
| Esophagus | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 |
| Stomach | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 |
| Duodenum | 11/11 | 11/11 | 11/11 | 11/11 | 11/11 |
| Ileum | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 |
| Colon | 1/12 | 1/12 | 1/12 | 1/12 | 1/12 |
| Pancreas | 0/7 | 0/7 | 0/7 | 0/7 | 0/7 |
| Liver | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 |
| Genitourinary system | | | | | |
| Kidney | | | | | |
| Glomeruli | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 |
| Proximal tubules | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 |
| Distal tubules | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 |
| Collecting ducts | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 |
| Bladder | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 |
| Testis | 0/9 | 0/9 | 0/9 | 0/9 | 0/9 |
| Breast | 8/8 | 8/8 | 8/8 | 8/8 | 8/8 |
| Ovary | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 |
| Skin | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 |
| Skeletal muscle | 7/7 | 0/7 | 0/7 | 0/7 | 0/7 |
| Endocrine system | | | | | |
| Thyroid | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 |
| Adrenal cortex/medulla | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 |


Fig. 4. Skeletal muscle. *A*, H&E-stained section. *B*, 7E11 immunohistochemical stain showing positive reaction in a subset of cells. *C*, PM2J004.5 immunohistochemical stain showing no reactivity.

note is the fact that the other internal domain-binding anti-PSMA mAb, PM2J004.5, did not bind skeletal muscle. Thus, it is likely that, in skeletal muscle, 7E11 uniquely cross-reacts with either a yet to be defined PSMA-like or a PSMA-unrelated molecule. The patchy distribution suggests that expression of this molecule may be restricted to either fast-twitch or slow-twitch muscle fibers.

Novel PSMA-based prostate cancer therapies, including anti-PSMA mAb-based therapies, are currently under investigation (35–37). The results of our study indicate that anti-PSMA mAb-based diagnostic and therapeutic modalities may be expanded to include antineovasculature targeting for a wide variety of malignant neoplasms. The importance of angiogenesis in neoplasia is well documented (38–40), and endothelial cell expression of PSMA appears highly restricted to tumor-associated neovasculature and may represent a novel target for antineovasculature based therapy. Recent *in vivo* localization by the $^{111}$In-labeled 7E11 mAb to a conventional (clear cell) renal cell carcinoma demonstrates the potential clinical utility of anti-PSMA mAbs in a nonprostate cancer (41). Enthusiasm for mAb-based therapy, however, must be tempered by the fact that PSMA is expressed in several benign tissue types; the potential side effects of anti-PSMA mAbs on these tissues *in vivo* is unknown. However, other mAbs that are currently in clinical trials or Food and Drug Administration-approved for clinical use, also are not tumor specific and bind antigens expressed in benign tissues (42, 43).

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*Advances in Brief*

# Constitutive and Antibody-induced Internalization of Prostate-specific Membrane Antigen[1]

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## Abstract

**Prostate-specific membrane antigen (PSMA) is a cell surface glycoprotein expressed predominantly by prostate cancer cells. We have characterized four monoclonal antibodies that bind to the extracellular domain of PSMA (Liu *et al.*, Cancer Res., 57: 3629–3634, 1997). Here we report that viable LNCaP cells internalize these antibodies. Laser scanning confocal microscopy reveals that the internalized antibodies accumulate in endosomes, and immunoelectron microscopy reveals that endocytosis of the PSMA-antibody complex occurs via clathrin-coated pits. In addition, a quantitative cell surface biotinylation assay demonstrates that PSMA is constitutively endocytosed in LNCaP cells and that anti-PSMA antibodies increase the rate of internalization of PSMA. These studies suggest that PSMA might function as a receptor mediating the internalization of a putative ligand. The availability of prostate-specific internalizing antibodies should aid the development of novel therapeutic methods to target the delivery of toxins, drugs, or short-range isotopes specifically to the interior of prostate cancer cells.**

## Introduction

PSMA[5] is the single most well-established highly restricted prostate epithelial cell membrane antigen (1–8). In contrast to other highly restricted prostate-related antigens such as prostate-specific antigen and prostatic acid phosphatase, which are secretory proteins, PSMA is an integral cell membrane protein. The PSMA gene has been cloned, sequenced (9), and mapped to chromosome 11q14 (10). One of the reasons for significant interest in PSMA is that it is ideal for *in vivo* prostate-specific targeting strategies. In addition to its prostate specificity (1–8), PSMA is expressed by a very high proportion of PCAs (1, 2, 4, 6, 7); expression is further increased in higher-grade cancers, metastatic disease (4, 6, 7), and hormone-refractory PCA (3, 6, 7). PSMA expression is modulated inversely by androgen levels (3, 6). Furthermore, PSMA expression has been found in tumor but not in normal vascular endothelium (7, 11), further broadening its interest and potential applications.

PSMA has been shown to have peptidase (12) and folate hydrolase (13) activity. Although it shares some homology with rat brain *N*-acetylated α-linked acidic dipeptidase (12) and the transferrin receptor (9), PSMA does not share the latter's internalization signal (9). The function of PSMA with respect to PCA and vascular endothelial cell biology and the direct correlation between its expression and increasing PCA aggressiveness remain intriguing and unclear.

Until recently, the only available mAb to PSMA was 7E11.C5 (14), which targets an epitope located within the short cytoplasmic tail of the molecule (15, 16). As a result, 7E11.C5 did not bind viable cells (11, 14, 16). We recently reported the development of four IgG mAbs that react with the external domain and define two distinct epitopes of PSMA ($PSMA_{ext1}$ and $PSMA_{ext2}$; Ref. 11). Because these mAbs are capable of binding viable PSMA-expressing cells, we have begun to use them in an effort to further understand the function of PSMA.

## Materials and Methods

**Antibodies and Reagents.** mAbs J591, J415, and J533 (all IgG1) and E99 (IgG3) to $PSMA_{ext}$ and mAb I56 (IgG1; negative control) to inhibin were generated as described previously (9). Purified mAb 7E11.C5 was a generous gift from Dr. Gerald P. Murphy (Pacific Northwest Research Foundation, Seattle, WA). Secondary antibody reagents conjugated with FITC and Texas Red were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

**Antibody Uptake.** LNCaP cells ($15 \times 10^4$) were plated on glass coverslips in 35-mm dishes and grown for 2–3 days before initiating the experiments. For the internalization assay, the cells were washed in RPMI 1640 containing 0.5% fatty acid-free BSA (RPMI-BSA) and incubated with mAbs J591, J415, 7E11.C5, or I56 at 4 μg/ml in RPMI-BSA at 37°C. When transferrin uptake was monitored, FITC-conjugated transferrin (Molecular Probes, Inc., Eugene, OR) was coincubated along with the respective antibody. The cells were washed and further processed for IF and confocal microscopy as described below. For immunoelectron microscopic detection of antibody uptake, the above-mentioned procedure was followed, except that the cells were grown directly on 35-mm culture dishes.

**IF and Laser Scanning Confocal Microscopy.** After primary mAb incubation, FITC-conjugated goat antimouse IgG [Jackson ImmunoResearch Laboratories; 1:100 in 1% BSA in PBS (pH 7.4)] was incubated for 30 min and washed extensively in 1% BSA in PBS. Slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA).

The internalization of FITC-conjugated transferrin and antibodies against PSMA was examined using a Phoibos 1000 laser scanning confocal microscope (Molecular Dynamics, Sunnyvale, CA) as described previously (16). To detect FITC- and Texas Red-labeled reagents simultaneously, samples were excited at 514 nm with an argon laser; the light emitted between 525 and 540 nm was recorded for FITC, and the light emitted above 630 nm was recorded for Texas Red. Serial optical sections of the monolayer were recorded at 0.4 μ intervals. A total of 30–40 horizontal (X-Y) confocal sections were obtained for each cell type and used to generate three-dimensional images using the Image Space software program (version 3.01; Molecular Dynamics) on an Iris Indigo Workstation (Silicon Graphics, Mountain View, CA).

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[5] The abbreviations used are: PSMA, prostate-specific membrane antigen; IEM, immunoelectron microscopy; IF, immunofluorescence; mAb, monoclonal antibody; PCA, prostate cancer.

**IEM.** After antibody incubation at 37°C for 2 h, the cells were washed in PBS in BSA, fixed for 20 min in cold methanol, and hydrated in PBS in BSA. Cells were then incubated for 1 h with 15-nm gold beads conjugated with goat antimouse IgG (Amersham Life Science, Inc., Arlington Heights, IL). After washing, the cells were fixed in 2.5% glutaraldehyde for 15 min, scraped gently, pelleted, and processed for IEM as described previously (11, 17). Electron micrographs were taken with a Joel 100 CX electron microscope.

**Cell Surface Biotinylation Assay for Endocytosis.** Biotinylation assays were performed as described by Bretscher and Lutter (18). Briefly, LNCaP cells ($60 \times 10^4$) were grown on polylysine (3%)-coated 60-mm dishes. Cells were washed in precooled PBS containing 1 mM each of calcium chloride and magnesium chloride. To biotinylate the cell surface proteins, cells were treated with the water-soluble, membrane-impermeable, cleavable biotin analogue sulfosuccinimidyl 2-(biotinamido) ethyl-1,3-dithiopropionate (NHS-SS-biotin; Pierce Chemical Co., Rockford, IL; 0.5 mg/ml) at 4°C for 20 min and then washed in RPMI-BSA. Two control dishes were kept on ice, whereas the other dishes were incubated at 37°C. The incubation was stopped at various times by transferring cells back to 4°C. After washing in 10% FCS in PBS, the cells were incubated twice for 20 min in reducing solution [310 mg of glutathione-free acid (Sigma, St. Louis, MO) dissolved in 17 ml of $H_2O$; 1 ml of 1.5 M NaCl, 0.12 ml of 50% NaOH, and 2 ml of serum were added just before use] to remove the residual cell surface exposed biotin. One control dish was reduced, and the second dish was not reduced, thereby serving as 0 and 100% biotinylation references, respectively. After washing, free sulfhydryl groups were quenched in iodoacetamide (5 mg/ml; Sigma) in BSA in PBS for 15 min. Cells were lysed, and PSMA was immunoprecipitated as described previously (11). Immunoprecipitates were analyzed by SDS-PAGE under nonreducing conditions. The gels were transferred to nitrocellulose membranes, and the blots were probed with $^{125}$I-streptavidin (Amersham), autoradiographed, and quantified using a densitometer (Molecular Dynamics).

## Results

**Staining of Viable Cells and Internalization of mAbs.** IF analysis of viable LNCaP cells incubated with mAbs J591, J533, E99, and J415 at 4°C showed distinct plasma membrane staining (data not shown), whereas mAb 7E11.C5 revealed no plasma membrane staining. Incubation of cells at 37°C with mAb J591 revealed labeling of both plasma membrane and intracellular vesicles (Fig. 1). After a 5-min incubation at 37°C, the labeling was detected primarily on the plasma membrane (Fig. 1*A*). At 20 min, distinct staining of intracellular vesicles was apparent (Fig. 1*B*), and at 180 min, intense labeling was observed in the juxtanuclear region, with sparse labeling throughout the cytoplasm (Fig. 1*C*). mAbs J415, J533, and E99 gave identical results (data not shown). mAb 7E11.C5 showed neither cell surface nor intracellular staining (data not shown) in these viable cells. These results indicate that mAbs to $PSMA_{ext}$ are internalized by viable PCA cells.

**Endosomal Localization of Internalized Antibodies.** To test whether internalized antibodies accumulate in endosomes, a simultaneous uptake of mAbs and FITC-labeled transferrin (an endosomal marker) was carried out. Laser scanning confocal microscopy revealed that internalized J591 (Fig. 2*A*) and transferrin (Fig. 2*C*) codistributed to a large extent (Fig. 2*E*), indicating that the internalized PSMA-antibody complex accumulates in endosomes. Control experiments with 7E11.C5 confirmed that this antibody is not internalized (Fig. 2, *B*, *D*, and *F*).

**IEM.** IEM of nonpermeabilized LNCaP cells at 4°C revealed mAb J591 binding to the extracellular side of the plasma membrane (11). IEM of viable LNCaP cells incubated with mAb J591 at 37°C for 10 min showed an accumulation of gold particles in clathrin-coated pits (Fig. 3, *A* and *B*) and in vesicles close to the plasma membrane (Fig. 3*C*). After a 2-h incubation at 37°C, vesicles containing gold beads were found in a juxtanuclear location (Fig. 3*D*). These findings indicate that mAb J591 internalization occurs via clathrin-coated pits in LNCaP cells.

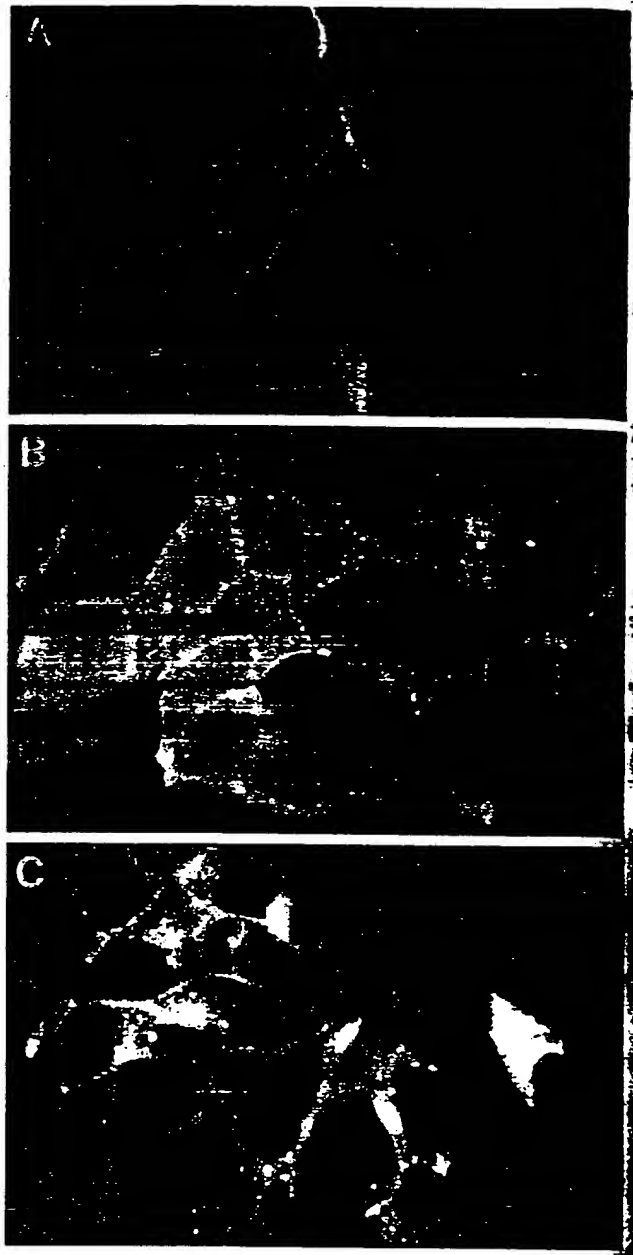


Fig. 1. Internalization of mAb J591 in LNCaP cells. Live cells were incubated with mAb J591 for 5 (*A*), 20 (*B*), and 180 (*C*) min. Cells were then permeabilized and stained with FITC-conjugated secondary antibody to visualize internalized mAb J591.

**Internalization of PSMA.** A cell surface protein biotinylation assay was developed to test whether PSMA is internalized in the absence of antibody, or whether antibody binding induces PSMA internalization. In this assay, a cleavable biotin analogue (NHS-SS-biotin) was used to label proteins exposed on the surface at 4°C. The return of surface biotinylated cells to a temperature of 37°C allows the internalization of the appropriate cell surface proteins with their biotin tag. The NHS-SS-biotin label is removed from noninternalized cell surface proteins through cleavage of the disulfide linkage with glutathione (18), whereas internalized biotinylated proteins are protected from this cleavage. The appearance of biotin-labeled protein that is resistant to glutathione reduction was taken as an indicator of inter-

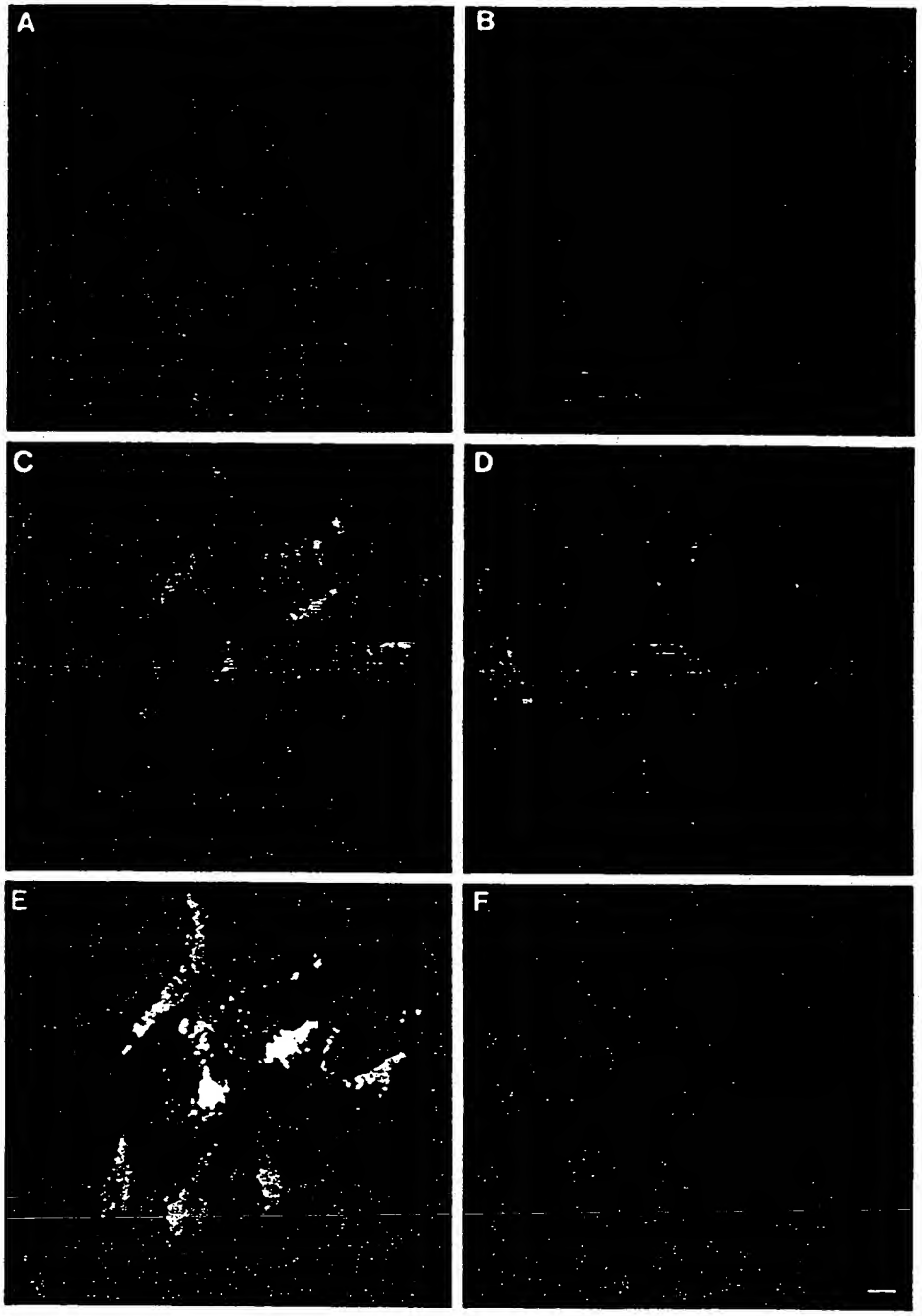


Fig. 2. Confocal microscope analysis of the internalization mAb J591. LNCaP cells were incubated with mAb J591 and FITC-conjugated transferrin (*A*, *C*, and *E*) or mAb 7E11.C5. and FITC-conjugated transferrin (*B*, *D*, and *F*) for 2 h and processed for IF as described in "Materials and Methods." mAbs J591 (*A*) and 7E11.C5. (*B*) were detected with a Texas Red-conjugated secondary antibody. FITC-conjugated transferrin uptake is shown (*C* and *D*). Images in *A* and *C* were merged to obtain the image in *E* (mAb J591 and FITC-conjugated transferrin colocalization, *yellow*). Images in *B* and *D* were merged to obtain the image in *F*, in which only transferrin uptake is seen because 7E11.C5. neither binds nor internalizes.

nalization. As shown in Fig. 4*A*, biotinylated PSMA is sensitive to glutathione reduction after labeling cells at 4°C (Fig. 4*A*, compare *Lanes 1* and *2*). However, with progressively longer incubation periods at 37°C, an increasing proportion of the labeled PSMA becomes increasingly resistant to reduction (Fig. 4*A*, *Lanes 3–6*). Quantitation of the blots revealed that 60% of the total cell surface PSMA was internalized (Fig. 4*C*) within 2 h.

When the biotinylated cells were incubated with 1 μg/ml mAb J591 at 37°C, a 3-fold increase in the rate of internalization of PSMA was observed (compare Fig. 4*B*, *Lanes 3* and *4* and Fig. 4*D*). A higher concentration of mAb J591 did not show any further increase in the internalization of PSMA (Fig. 4*B*, *Lanes 5–7*; Fig. 4*D*). Similar results were obtained with monovalent Fab fragments (data not shown).

## Discussion

The prostate-restricted nature of PSMA, coupled with the direct association between the level of PSMA expression and increasingly aggressive disease (4), implies a potentially important role for PSMA in PCA biology. The importance of understanding the function of PSMA is further stimulated by its expression in vascular endothelium specifically supplying cancers but not in normal, resting endothelium (7, 11). In the past, investigating PSMA function has been compromised because the sole antibody to PSMA reacted with a cytoplasmic epitope of the molecule and therefore bound only to cells that were permeabilized or dead (11, 14, 16). Our recent development of mAbs to the extracellular domain of PSMA and their demonstrated ability to

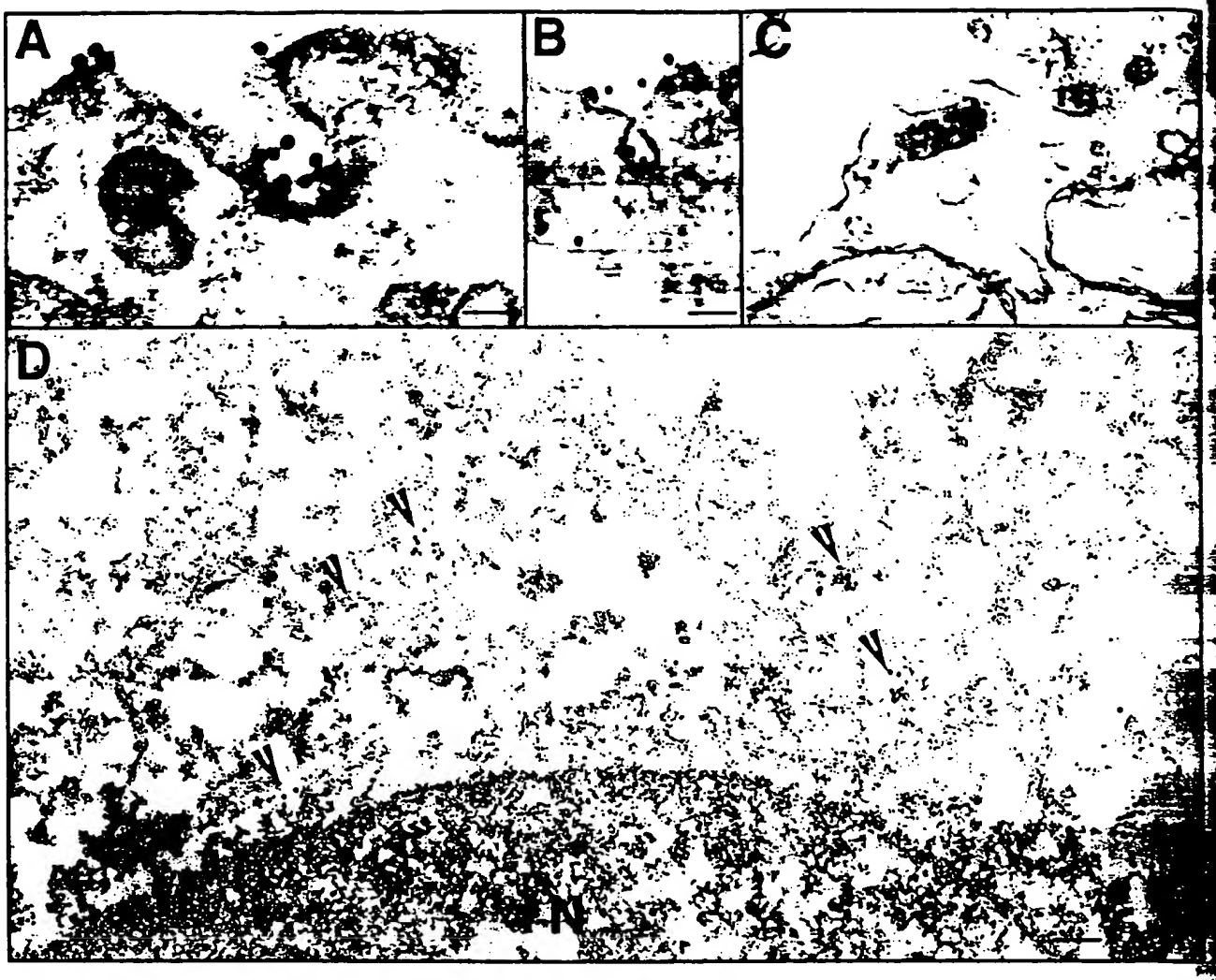


Fig. 3. IEM of the internalized mAb J591 in LNCaP cells. Cells were incubated with J591 at 37°C for 10 min (*A–C*) or 2 h (*D*) and processed for immunogold labeling as described in "Materials and Methods." Note the accumulation of gold particles in clathrin-coated vesicles (*A* and *B*) and in vesicles proximal to the plasma membrane (*C*). At 2 h, note the accumulation of gold particles in a juxtanuclear region (*arrowheads*). *N*, nucleus. *Bars* represent 34 (*A*), 65 (*B* and *C*), and 85 nm (*D*), respectively.

bind viable cells (11) have provided a means to study the function of PSMA.

In this study, we demonstrate by a combination of microscopical and biochemical techniques that PSMA and mAbs to $PSMA_{ext}$ are internalized by LNCaP cells. Confocal microscopy and IEM reveal that PSMA-mAb complexes are endocytosed via clathrin-coated pits (Figs. 2 and 3). A quantitative cell surface biotinylation assay demonstrates that PSMA is constitutively internalized in the absence of antibody binding. At 20 min, 15% of the total biotinylated surface PSMA is internalized (Fig. 4*C*). The proportion of surface PSMA internalized increases to 60% at 60 min and remains fairly constant at that level for 240 min thereafter, when the assay was terminated. The stability of the labeled PSMA for a period of over 6 h (data not shown) indicates that PSMA degradation during this period is minimal. Internalization of only 60% of the total labeled surface PSMA may be explained by the recycling of internalized, biotinylated PSMA back to the cell surface,[6] where it would be reduced and rendered undetectable in this assay.

Constitutive internalization of PSMA may reflect the recycling structural protein through a plasma membrane location or may mediated by the binding of a ligand. Whereas the finding that PSMA antibody significantly increases the rate of internalization PSMA is consistent with the latter ligand receptor-type function, does not necessarily indicate that PSMA has a transport function. In the presence of mAb to $PSMA_{ext}$, the rate of internalization of PSMA increased up to 3-fold in a dose-dependent manner, reaching a maximum rate at an antibody concentration of 1–2 μg/ml (Fig. 4). A similar increase in the internalization rate has been shown for epidermal growth factor and its ligand (19).

It is well established that many ligands and their transmembrane receptors are internalized via clathrin-coated pits (receptor-mediated endocytosis) (20). The formation of antigen-antibody complexes on the cell surface often results in internalization through a pathway closely resembling the receptor-mediated endocytosis of peptide hormones, growth factors, and other natural ligands (21). Based on our findings, we hypothesize that PSMA may have a transport function an as yet unidentified ligand. The baseline internalization rate PSMA may indicate that PSMA may internalize in the absence

[6] H. Liu, R. Rahmati, and N. H. Bander, unpublished observations.

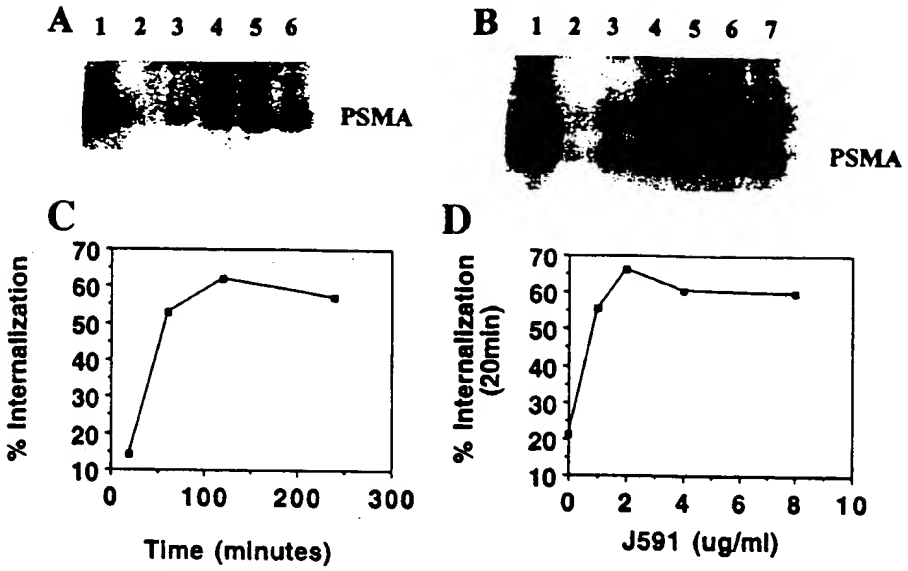


Fig. 4. mAb-independent (*A* and *C*) and mAb-induced (*B* and *D*) internalization of PSMA. The cell surface was biotinylated at 4°C with NHS-SS-biotin and then transferred to 37°C. Biotin was reduced at 4°C with glutathione as described in "Materials and Methods." PSMA was immunoprecipitated, run on a 10% SDS-PAGE, and detected with $^{125}$I-streptavidin. Lanes in *A* are as follows: *Lane 1*, incubation at 40°C with no reduction (100% control); *Lane 2*, incubation at 4°C followed by reduction (0% control); and *Lanes 3–6*, incubation at 37°C for 20, 60, 120, and 240 min followed by reduction. Note the complete reduction of biotinylated PSMA in *Lane 2* compared with *Lanes 3–6*, indicating that PSMA is internalized. Densitometric quantitation (*C*) revealed that approximately 60% of the total cell surface PSMA is internalized. mAb J591 induced the internalization of PSMA (*B* and *D*). LNCaP cells were biotinylated at 4°C and incubated with different amounts of mAb J591 for 20 min at 37°C. Lanes in *B* are as follows: *Lane 1*, incubation at 4°C with no reduction; *Lane 2*, incubation at 4°C followed by reduction; and *Lanes 3–7*, incubation at 37°C with 0, 1, 2, 4, and 8 μg/ml J591 followed by reduction. Note the increased uptake of PSMA in the presence of 1 μg/ml J591 (*Lane 4*) compared with *Lane 3*, in which no antibody is present. Increasing the mAb J591 concentration above 1 μg/ml did not increase the uptake, indicating a saturation of PSMA uptake. Densitometric quantitation (*D*) revealed that 1 μg/ml mAb J591 increased the uptake of PSMA by approximately 3-fold.

ligand, or, alternatively, that the PSMA ligand may be present in the culture medium. Similarly, mAb or mAb fragments act as a surrogate ligand, inducing an increased rate of internalization. The internalization pattern seen in this study may have been influenced or modified by the presence of mAb (22) and may not reflect the natural internalization pattern.

The targeting of most receptors to coated pits and their traffic through the endocytic compartment are thought to be mediated by a specific internalization motif in the cytoplasmic domain of the receptor (20). The first well-characterized internalization motifs of several receptors, including the transferrin receptor, mannose-6-phosphate receptor, asialogylcoprotein receptor, polymeric immunoglobulin receptor, and others, are all tetrapeptides (Tyr-*X*-Arg-Phe) having an aromatic residue in the fourth position of the sequence (23). The cytoplasmic tail of PSMA lacks a sequence similar to the Tyr-*X*-Arg-Phe motif (9). Another signal is the dileucine motif, for which the only known requirement is the presence of two consecutive leucines or a leucine-isoleucine pair. The dileucine motif has been shown to mediate internalization and targeting to endosomes and lysosomes (24). A dileucine motif is present in the cytoplasmic tail of PSMA. Experiments are under way to confirm the dileucine internalization motif of PSMA. Interestingly, whereas PSMA is 85% homologous to a rat brain neuropeptidase (24), this homology is located primarily at the COOH-termini and declines to less than 50% homology at the $NH_2$-termini. Furthermore, rat brain neuropeptidase lacks both the Tyr-*X*-Arg-Phe and dileucine motifs (25) and presumably does not internalize. Therefore, the highly restricted expression of PSMA becomes increasingly PCA-specific via different mechanisms. For example, at the mRNA level, normal and benign hyperplastic prostate epithelia predominantly express the cytosolic PSM′ splice variant without a significant membrane-expressed component, whereas in PCA, the membrane form predominates by 10–100-fold (26). Another form of functional specificity is demonstrated in rat brain astrocytes (25); although there is expression of a homologous neuropeptidase, this neuropeptidase is presumably not internalized as is PSMA in PCA cells.

The property of mAbs to $PSMA_{ext}$ to be internalized in PCA cells adds another dimension to their *in vivo* therapeutic potential. In addition to selective/specific binding to the PCA cell surface, the mAb or fragment would be internalized into the targeted cells, providing direct access to the neoplastic cell machinery. As such, this property opens up options such as the use of toxin or drug conjugates. Similarly, the juxtanuclear location of the internalized vesicles should increase the potency of mAb-α particle conjugates by improving the incident angle of the isotope and the target DNA.

Lastly, although mAb may function as a surrogate ligand, the question remains as to the identity of the putative natural ligand of PSMA. Troyer *et al.* (16) noted a $M_r$ 40,000 band that coimmunoprecipitated with PSMA that they identified as *S*-glutamic oxalacetic transaminase. We have not been able to demonstrate the binding of *S*-glutamic oxalacetic transaminase to PSMA (data not shown). Further study will be required to define the putative natural ligand, which, in turn, may shed additional light on the role of PSMA in cancer biology and tumor angiogenesis. The natural ligand, if similarly restricted in its tissue receptor binding profile, may substitute for the mAb in a targeted therapy approach.

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